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Studies on Plant Growth Substances

Part XIII*. Plant Growth Activity of Substituted 1-Naphthoic Acid Derivatives**

By Koichi KOSHIMIZU, Toshio FUJITA, Tetsuo MITSUI and Jiro KATO***

Department of Agricultural Chemistry, Kyoto University, Kyoto

Received July 8, 1959

1-Naphthoic acid derivatives substituted with chlorine, bromine, methyl or nitro groups at various positions of the ring were assayed for their activities in the pea straight-growth and the callus formation tests. Their growth activities are discussed from two points of view, i.e. the spatial structure hypothesis of Veldstra and the *ortho* reaction hypothesis advanced for benzoic acid derivatives proposed by Hansch and Muir. It appears that a combination of these two views are appropriate for the present authors' results.

INTRODUCTION

The relationship between chemical structure and plant growth activity has not been investigated with substituted naphthoic acid derivatives so extensively as with the series of substituted benzoic acids[1]. It is reasonable expected that these two series of compounds, in which a carboxyl group is directly bound to the aromatic ring, are more or less similar to each other in their physical and chemical properties, — 1-(or 2-) naphthoic acids corresponding to 2,3-(or 3,4-) dialk(en)yl benzoic acids. Hence, studies of substituted naphthoic acids may contribute to the understanding of the structural requirement for growth promoting activity. From this viewpoint 1-naphthoic acid derivatives substituted with a chlorine, bromine, methyl or nitro group at various positions were prepared and growth activity was measured.

EXPERIMENTAL

Methods of bioassay

Details of the methods of the pea straight-growth test and the callus formation test have previously been given[2,3]. The former is appropriate for testing weakly active growth substances.

Preparation of compounds*.

3-, 4- and 6-Nitro-1-naphthoic acids were prepared by the method of Leuck et al.[4]. 3-Nitro acid, m.p. 267° (reported 270.5~271.5°[4]). *Anal.* Found: C, 60.93; H, 3.53. Calcd. for $C_{11}H_7O_4N$: C, 60.83; H, 3.25. 4-Nitro acid, m.p. 223~224° (reported, 226°[4]). *Anal.* Found: C, 60.71; H, 3.27. 6-Nitoro acid, m.p. 223° (reported, 227~227.5°[4]). *Anal.* Found: C, 60.54; H, 3.43.

5- and 8-Nitro-1-naphthoic acids; 1-Naphthoic acid was nitrated with concentrated nitric acid, and 5- and 8-nitro acids were separated from the mixture by recrystallization with alcohol[5]. 5-Nitro acid, m.p. 239° (reported, 239°[5]). *Anal.* Found: C, 60.73; H, 3.20. Calcd. for $C_{11}H_7O_4N$: C, 60.83; H, 3.25. 8-Nitro acid, m.p. 217° (reported, 215°[5]). *Anal.* Found: C, 60.57; H, 3.18.

2-Chloro-1-naphthoic acid: 2-Hydroxy-1-naphthoic acid derived from 2-naphthol by means of the Kolbereaction[6] was treated with phosphorus pentachloride, and resulting chloro-naphthoic acid chloride was converted to the acid[7], m.p. 155∼156° (reported, 152∼153°[7], 154∼155°[8]). *Anal.* Found: C, 64.17; H, 3.69. Calcd. for $C_{11}H_7O_2Cl$: C, 63.94; H, 3.41.

---

* Part XII by M. Inaba and T. Mitsui, This Bulletin, **20**, 42 (1956).

** A part of this work was presented at the annual meeting of Agricultural Chemical Society of Japan, Tokyo, April 10, 1959.

*** Department of Botany, Kyoto Univ., Kyoto.

[1] *e.g.* H. Veldstra, *Ann. Rev. Plant Physiol.*, **4**, 151 (1953).

[2] J. Kato, *Mem. Coll. Sci. Univ. Kyoto*, **B 20**, 32 (1951).

[3] J. Kato, *Physiol. Plant.*, **11**, 200 (1958).

* All melting points are uncorrected.

[4] G. J. Leuck, R. P. Perkins and F. C. Whitmore, *J. Am. Chem. Soc.*, **51**, 1831 (1929).

[5] A. G. Eckstrand, *J. Prakt. Chem.*, **(2)**, **38**, 155, 241 (1888).

3-, 4-, 5- and 6-Chloro-1-naphthoic acids were prepared according to the Sandmeyer-reaction from the corresponding amino acids obtained by reduction of the nitro acids[5]. 3-Chloro acid, m.p. 221° (reported, 223°[9]). *Anal.* Found: C, 63.76; H, 3.66; Cl, 17.08. Calcd. for $C_{11}H_7O_2Cl$; C, 63.94; H, 3.41; Cl, 17.17. 4-Chloro acid, m.p. 220∼221° (reported 225°[9]). *Anal.* Found; C, 64.07; H, 3.64; Cl, 16.96. 5-Chloro acid, m.p. 247° (reported, 245°[5]). *Anal.* Found: C, 64.09; H, 3.40; Cl, 17.04. 6-Chloro acid, m.p. 213° (reported, 215.8∼216.2°[10]). *Anal.* Found: C, 63.89; H, 3.64.

3-, 4-, 5- and 6-Bromo-1-naphthoic acids were prepared from the amino acids, essentially in the same way as described above by means of the Sandmeyer-reaction. 3-Bromo acid, m.p. 231.5∼232.5° (reported, 236°[9]). *Anal.* Found: C, 52.56; H, 2.82; Br, 32.18. Calcd. for $C_{11}H_7O_2Br$: C, 52.62; H, 2.81; Br, 31.83. 4-Bromo acid, m.p. 220∼221° (reported, 221°[9]). *Anal.* Found: C, 52.70; H, 3.03. 5-Bromo acid, m.p. 256∼257° (reported, 246°[5], 262°[10]). *Anal.* Found; C, 52.55: H, 3.07. 6-Bromo acid, m.p. 183∼184°. *Anal.* Found: C, 52.43; H, 2.83.

8-Chloro- and 8-bromo-1-naphthoic acids were prepared by treating with chlorine or bromine anhydro-8-hydroxymercuri-1-naphthoic acid[11] obtained from naphthalic acid[4]. 8-Chloro acid, m.p. 171∼172° (reported, 170∼170.5°[11]). *Anal.* Found: C, 63.63; H, 3.70; Cl, 17.07. Calcd. for $C_{11}H_7O_2Cl$: C, 63.94; H, 3.41; Cl, 17.17. 8-Bromo acid, m.p. 176∼178° (reported, 176∼179°[11]). *Anal.* Found: C, 52.83; H, 2.85; Br, 32.08. Calcd. for $C_{11}H_7O_2Br$: C, 52.62; H, 2.81; Br, 31.83.

2-Methyl-1-naphthoic acid was obtained by carbonation with carbon dioxide from 1-magnesium bromide derivative of 2-methylnaphthalene[12], m.p. 125∼126° (reported, 126∼127°[12]). *Anal.* Found: C, 77.50; H, 5.61. Calcd. for $C_{12}H_{10}O_2$; C, 77.40; H, 5.41.

3-Methyl-1-naphthoic acid; 3-Methyl-1-bromonaphthalene prepared according to Fischer et al.[9] was converted to 3-methyl-1-naphthoic acid in the same way as described above, m.p. 171∼172° (reported, 171∼172°[9]). *Anal.* Found: C, 77.34; H, 5.58. Calcd. for $C_{12}H_{10}O_2$: C, 77.40; H, 5.41.

4-Methyl-1-naphthoic acid was prepared in the same way as described in case of 2-methyl acid[12], m.p., 175∼176° (reported, 175°[12]). *Anal.* Found: C, 77.17; H, 5.65. Calcd. for $C_{12}H_{10}O_2$: C, 77.40; H, 5.41.

6-Methyl-1-naphthoic acid was prepared by the reaction of *α*-furoic acid with toluene in the presence of anhydrous aluminum chloride[13], m.p. 176∼177° (reported, 176.5∼177°[13]). *Anal.* Found: C, 77.66; H, 5.43. Calcd. for $C_{12}H_{10}O_2$: C, 77.40; H, 5.41.

8-Methyl-1-naphthoic acid was prepared by the Clemmensen reduction of naphthaldehydic acid[14], m.p., 153° (reported, 153°[14]). *Anal.* Found: C, 77.23; H, 6.60. Calcd. for $C_{12}H_{10}O_2$: C, 77.40; H, 5.41.

RESULTS AND DISCUSSION

Activities of halogen (chlorine and bromine) substituted 1-naphthoic acids on the straight-growth of pea stem section are shown in Figs. 1 and 2. The growth activity is intensified by the substitution at positions of 2 or 8, whereas it is reduced by substitution at one of the other positions, 3- and 5-halogeno-acids being almost inactive. Essentially the same effects of halogen substitutions were also found in callus forming activity (Table I).

The order of methyl substituted 1-naphthoic acids with respect to their growth activities is similar to that of the halogeno-acid series (Fig. 3 and Table I). In this case, however, unlike 2-chloro-acid, 2-methyl acid is less active than the parent acid. All the nitro acids are less active than the parent acid, as shown in Fig. 4 and Table I.

Veldstra et al.[1,15,16] have affirmed that the essential requirements for a growth promoting substance are as follows: (a) the HL-balance

---

6) Am. Pat. 2,132,357; *Chem. Zentr.*, **1938**, II, 4312.

7) H. Rabe, *Ber.*, **22**, 392 (1889).

8) H. Veldstra, *Rec. Trav. Chim.*. **71**, 15 (1952).

9) A. Fischer, W. J. Mitchell, G. S. Ogilvie, J. Packer, J. E. Packer and J. Vaughan, *J. Chem. Soc.*, **1958**, 1426.

10) T. L. Jacobs, S. Winstein, R.B. Henderson, J. Bond, J.W. Ralls, D. Seymour and W. H. Florsheim, *J. Org. Chem.*, **11**, 229 (1946).

11) F. C. Whitmore and A. L. Fox, *J. Am. Chem. Soc.*, **51**, 3363 (1929).

12) F. Mayer and A. Sieglitz, *Ber.*, **55**, 1835 (1922).

13) C. C. Price, E. C. Chapin, A. Goldman, E. Krebs and H. M. Shafer, *J. Am. Chem. Soc.*, **63**, 1857 (1941).

14) R. C. Fuson and G. Munn, *J. Am. Chem. Soc.*, **71**, 1870 (1949).

15) H. Veldstra, *Enzymologia*, **11**, 97, 137 (1944).

16) H. Veldstra and H. L. Booij, *Biochim. Biophys. Acta*, **3**, 278 (1949).

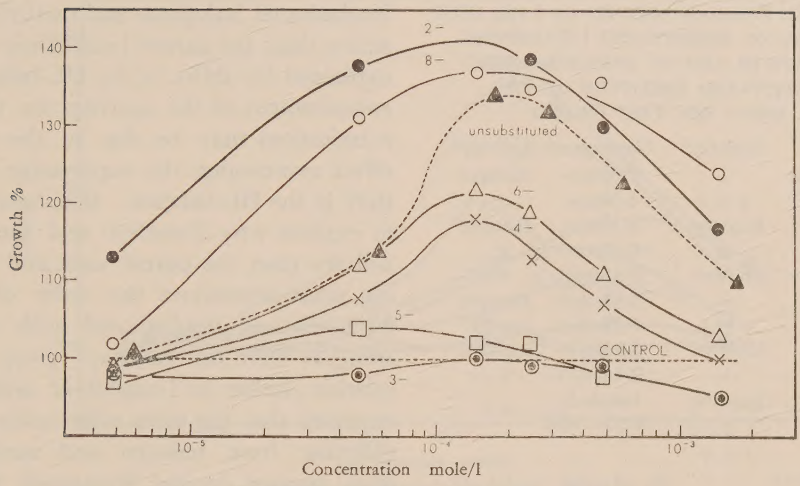

FIG. 1. Activity of Chloro-1-Naphthoic Acids in the Straight-Growth of Pea Stem Section

Each point in Figs. 1~4 is the average of two runs of experiments, 10 section pieces being used for each lot in each run.

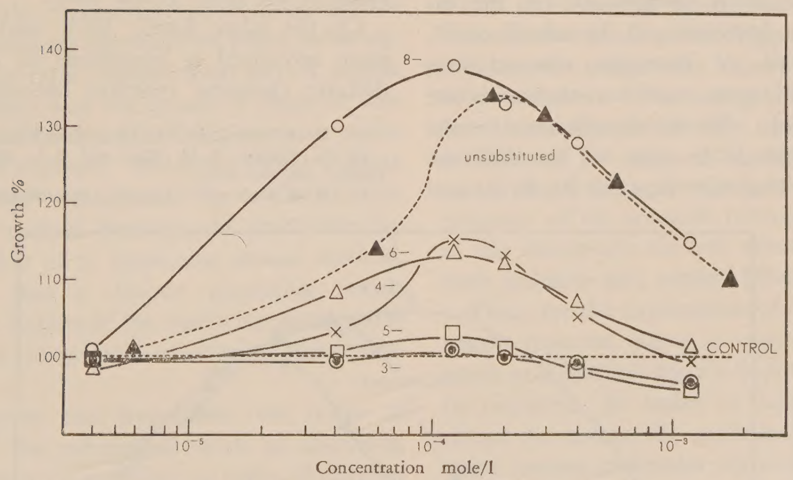

FIG. 2. Activity of Bromo-1-Naphthoic Acids in the Straight-Growth of Pea Stem Section

between the basal lipophilic ring system and the hydrophilic substituent such as the carboxyl group and, (b) definite spatial position of the hydrophilic substituent in relation to the ring system. From this viewpoint Veldstra[8,17] has stated that the activities of substituted benzoic acids are dependent on a non-flat structure caused by the *ortho* substituent of such a size that the carboxyl group is not masked out. On the same basis he has considered that the weak activity of 1-naphthoic acid is attributable to a structure in which the carboxyl group is slightly

---

[17] H. Veldstra, "The chemistry and mode of action of plant growth substances", Butterworths Scientific Publications, London, 1956, p. 117.

TABLE I. CALLUS FORMING ACTIVITY OF 1 PER CENT LANOLIN PASTE OF SUBSTITUTED 1-NAPHTHOIC ACID DERIVATIVES AND OF INDOLE-3-ACETIC ACID, DECAPITATED EPICOTYLE OF *Vicia Faba* L. BEING THE TEST OBJECT

Compound	Activity*	Compound	Activity*
1-Naphthoic acid	+	3-Nitro-	Inactive
2-Chloro-	+++	4-Nitro-	±
3-Chloro-	Inactive	5-Nitro-	Inactive
4-Chloro-	±	8-Nitro-	±
5-Chloro-	Inactive	2-Methyl-	+
6-Chloro-	+	3-Methyl-	Inactive
8-Chloro-	+++	4-Methyl-	Inactive
3-Bromo-	Inactive	6-Methyl-	Inactive
4-Bromo-	±	8-Methyl-	++
5-Bromo-	Inactive	Indole-3-acetic acid	++
6-Bromo-	+		
8-Bromo-	+++		

* Order of activity: +++, ++, +, ±.

hindered by the spatial influence of the *peri* position, and that the enhanced activities of 2-chloro-, 8-halogeno- (chloro-, bromo- and iodo-) and 8-methyl acids result from the carboxyl group being twisted more strongly by the influence of the neighbouring bulky substituent17).

The present results concerning the activities of 2-chloro-, 8-halogeno- and 8-methyl-acids are in good agreement with the experimental results reported by Veldstra in spite of the different test methods. The fact that the 3-, 4-, 5- and 6-substituted halogeno- and methyl-acids are less active than the parent 1-naphthoic acid may be explained by shifts in the HL-balance, and the enhancement of the activity by the 2- and 8-substitutions may be due to the strong steric effect overcoming the suppressive effect of the shift in the HL-balance. It is, however, difficult to explain why 2-methyl acid shows a weaker activity than the parent acid, and also to point out what determines the order of activities of 3-, 4-, 5- and 6-substituted acids all of which seems to have the carboxyl group in a spatial relation similar to 1-naphthoic acid. It can be expected that the nitro substitution gives effects differing from halogen and methyl substitutions, because, by the firstnamed, the molecule becomes more lipophilic so as to make the HL-balance improper, and the carboxyl group may be less twisted in the 8-nitro acid than in the 8-halogeno- and 8-methyl acids according to the twist of the nitro group itself.

On the other hand, Muir and Hansch18~20) have advanced a hypothesis in concern of a definite chemical reaction between the auxin

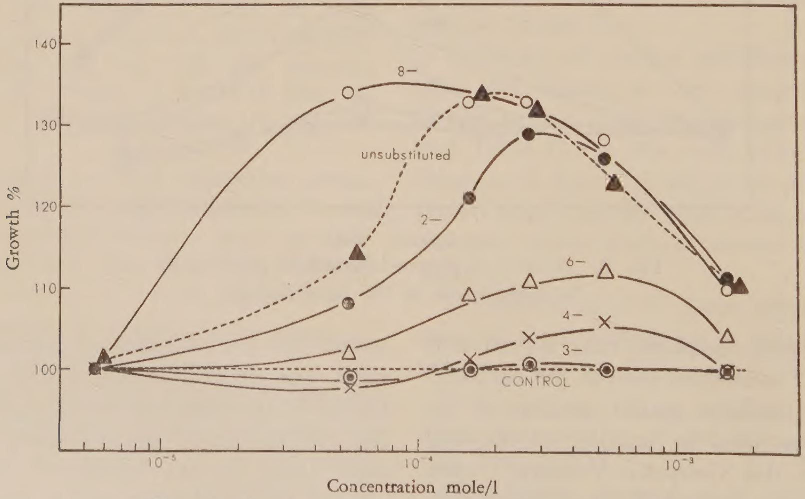

FIG. 3. Activity of Methyl-1-Naphthoic Acids in the Straight-Growth of Pea Stem Section

---

18) R. M. Muir and C. Hansch, *Plant Physiol.*, **26**, 369 (1951).

19) C. Hansch, R. M. Muir and R. L. Metzenberg, *Plant Physiol.*, **26**, 812 (1951).

20) R. M. Muir and C. Hansch, *Plant Physiol.*, **28**, 218 (1953).

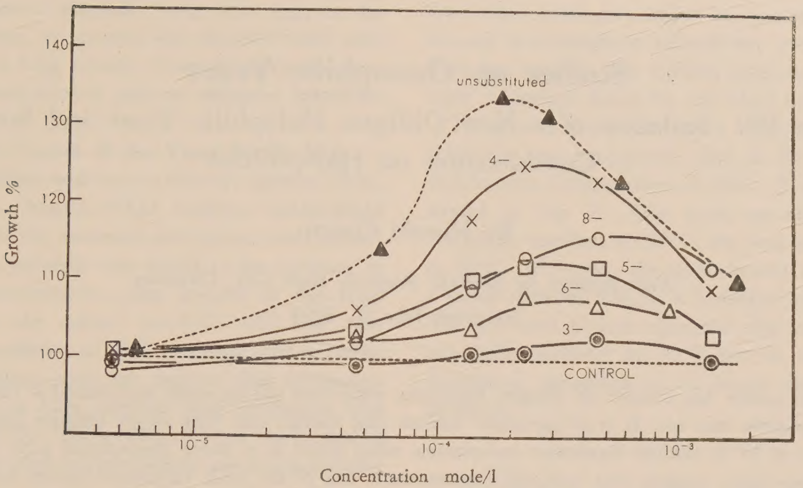

FIG. 4. Activity of Nitro-1-Naphthoic Acids in the Straight-Growth of Pea Stem Section

molecule and the cellular proteinous material. According to the above authors, at least one of the *ortho* positions of the ring to the point of attachment of the carboxyl group must be capable of reaction with an electron-rich plant substrate. Therefore, the *ortho* position must have a proper electron density and the group or atom at that position should be capable of displacement under conditions of reaction. Recently, Fukui, Nagata and Yonezawa[21] calculated the π-electron distribution of various benzoic acid derivatives by the application of a molecular orbital method, and found that a distinct paralellism exists between the indices of the reactivity at the *ortho* position and the growth activities of these compounds.

According to this hypothesis, the order of activities of the substituted acids as shown in Figs. 1, 2, 3 and 4 may reflect an order of electron density (or chemical reactivity) at a definite position of the naphthalene ring. Although the 2-position of 1-naphthoic acid, which corresponds to the *ortho* position of benzoic acid, may be capable of displacement by an electron-rich plant substrate, substitution of this position by a methyl group may lock up such a reaction. This assumption may explain the fact that 2-methyl acid is less active than the 2-chloro-acid. Since, however, the 2-methyl acid has an activity which is only slightly lower than the parent acid, it should be admitted that the spatial requirement suggested by Veldstra, or some other unknown factors may have some effect on the activity of this acid. The relatively low activities of the nitro acid series may also be comprehended by the strong electron attracting property of the aromatic nitro group in contrast to the mesomeric electron releasing property of both halogen and methyl groups.

Thus, for the explanation of the experimental results reported above a combination of Veldstra's and Hansch-Muir's hypotheses appears to be required. In order to hold ground of the above discussions the authors have determined more precise molecular structures of the acids in question and are examining the chemical reactivity indices of definite positions of the naphthalene ring. The results will be reported elsewhere.

The authors wish to express their appreciation to Prof. Joji Ashida for his interest and encouragement in the course of this work. They are also grateful to the Ministry of Education, for defraying a grant-in-aid.

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21) K. Fukui, C. Nagata and T. Yonezawa, *J. Am. Chem. Soc.*, **80**, 2267 (1958).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 226~230, 1960]

Studies on Osmophilic Yeasts

Part IX. Isolation of a New Obligate Halophilic Yeast and Some Consideration on Halophilism

By Hiroshi Ōnishi

Noda Institute for Scientific Research, Noda City, Chiba-ken

Received July 27, 1959

The author has isolated an obligate halophilic yeast from old soy-mash and named it *Torulopsis halonitratophila nov. sp.* It is of particular interest that though this yeast shows obligate halophilic property at 30° it exhibits facultative halophilism when tested at a lower temperature such as 20°. This observation suggests that cultural temperature is one of the most important factors for halophilism.

INTRODUCTION

Flannery[1] proposed the following classification for microorganisms according to their growth responses in media containing salts.

I. Non-halophiles

A. Salt-sensitive: Grow only in media containing less than 2% salt.

B. Salt-tolerant: Grow best in media containing less than 2% salt, but are also able to grow in media containing salt at concentrations of more than 2%.

II. Halophiles

A. Facultative: Will grow in media containing salt at concentrations less than 2%, but grow best in media containing more than 2%.

B. Obligate: Grow only in media containing salt at concentrations exceeding 2%.

As far as bacteria are concerned, a number of microorganisms which are obligately halophilic have been recognized[2,3]. However, no obligate halophilism has yet been found in yeast, only salt-tolerant types being so far described.

In the present paper, the isolation of an obligate halophilic yeast from old soy-mash will be described. This yeast has been named *Torulopsis halonitratophila nov. sp.* according to taxonomic studies. Conditions affecting halophilism were also examined, and it was revealed that incubation temperature is the most important factor.

EXPERIMENTAL AND RESULTS

[I] Isolation of Yeast from Soy-mash.

An appropriate dilution of a 12 month-old soy mash was plated on koji agar containing 18% NaCl. From colonies appearing on the plates after 10 days' incubation at 30°C, a strain of yeast M 3 was obtained. Subculture of the yeast was purified through single colony isolation on koji agar containing 18% NaCl.

[II] Halophilic Property of the Yeast Strain M 3.

As this yeast could originally not grow in an ordinary koji extract at 30°C, it seemed that this yeast required the addition of sodium chloride in the medium for growth. In order to acclimatize the yeast in the absence of salt, successive subcultures were carried out in media containing salt of a gradually decreasing concentration. The first medium contained 18% NaCl and cells grown in this medium were inoculated in the second medium containing 15% NaCl. In each subculture, concentration of NaCl in the medium was 3% lower than concentration of the preceeding subculture. The final medium contained no NaCl. It was observed that this yeast is able to grow in media containing more than 6% NaCl. In the 3% NaCl medium, however, growth was observed after a markedly long time lag; in the NaCl-free medium, no growth was observed even after incubation for a long period. It is of particular interest that this yeast showed such an obligated halophilic property.

---

[1] W. L. Flannery, *Bact. Rev.*, **20**, 49 (1956).

[2] W. L. Flannery, R. N. Doetsch and P. A. Hansen, *J. Bact.*, **64**, 713 (1952).

[3] J. Robinson and N. E. Gibbons, *Can. J. Botany*, **30**, 147 (1952).

〔III〕 Growth Curves of the Yeast Strain M 3.

The basal medium used was as follows; glucose 10%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2 \cdot 2H_2O$ 0.01%, NaCl 0.01%, casamino acid 0.4%, yeast extract 0.1%. Sodium chloride was added to the medium at 6 different concentrations. One loopful of the yeast cells taken from the culture on 6% NaCl koji agar after 7 days' incubation at 30°C was suspended in sterile physiological saline, then one drop of this suspension was inoculated to each medium and incubation was carried out at 30°C. The growth of yeast was measured turbidimetrically with a photoelectric spectrophotometer at 660 mμ.

The growth curves of this yeast in media at various concentrations of NaCl at 30°C are illustrated in Fig. 1. The optimum concentration of NaCl for growth of this yeast was found to be about 9∼13%.

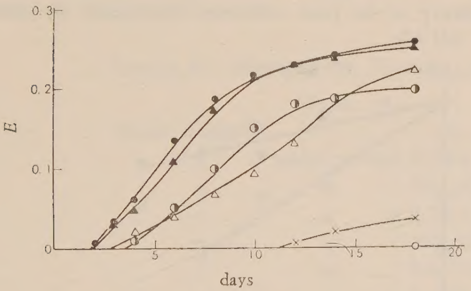

FIG. 1. Growth Curves at 30°C of the Yeast Strain M 3 in Media of Various NaCl Concentrations

○: NaCl 0%
×: NaCl 3%
△: NaCl 6%
●: NaCl 9%
▲: NaCl 13%
◑: NaCl 18%

In the previous paper[4], it was reported that *Saccharomyces rouxii*, which being a typical salt-tolerant type displays an important role in soy-brewing, is able to grow at a high temperature such as 40°C in media with a high concentration of salts or sugars, although the yeast failed to grow in the ordinary plain medium at the same temperature. In other words, *Sacch. rouxii* was transformed into apparently an obligate halophilic type when tested at a higher temperature as 40°C, but showed non-halophilic salt-tolerant property at 30°C. This fact suggests that cultural temperature may be the most important factor for microbial halophilism.

The growth of the yeast strain M 3 was examined at a relatively low temperature such as 20°C in the media with various concentrations of NaCl. The results are illustrated in Fig. 2. This yeast was able to grow in a NaCl-free medium at 20°C but was not able to grow at 30°C. However, the yeast showed better growth in a saline medium than in a NaCl-free medium at 20°C. The fact soon became clear that this yeast has obligate halophilic property at 30°C but is transformed into facultative halophilus at a lower temperature such as 20°C.

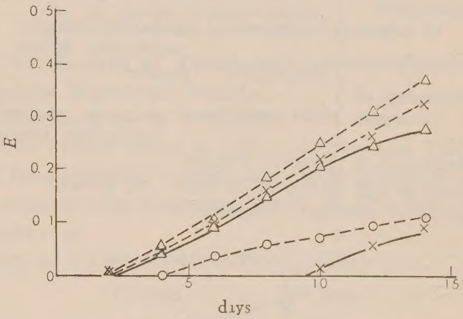

FIG. 2. Influences of Temperature on Growth of the Yeast Strain M 3 in the Ordinary Plain and the Salt-containing Media.

○: NaCl 0%
×: NaCl 3%
△: NaCl 6%
Solid line: 30°C
Broken line: 20°C

[IV] Taxonomic Studies on Yeast strain M 3.

The methods employed and key to the classification referred here were similar to those outlined in the monograph by Lodder and Kreger-van Rij[5]. Minor modifications and additions as described in the previous report[6] were made.

The experiments were carried out at an optimum condition for growth of this yeast, i. e. it was cultivated at 30°C in 6% NaCl media. The results are shown in Table I. The results were consistent with those obtained from experiments at 20°C using NaCl-free media.

According to the characteristics shown in Table I, this

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[4] H. Ōnishi, This Bulletin, **23**, 351 (1959).

[5] J. Lodder and N.J.W. Kreger-van Rij, The Yeasts, a Taxonomic Study (1952).

[6] H. Onishi, This Bulletin, **21**, 151 (1957).

TABLE I. MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE YEAST STRAIN M 3

Vegetative cells after 10 days' incubation in 6% NaCl koji extract, at 30°C: Cells are oval, ellipsoidal and usually measure $(2.5\sim3)\,\mu\times(4\sim5)\,\mu$

Growth on 6% NaCl Koji agar, after 2 weeks at 30°C: The streak culture is greyish brown, dull glistening, raised and almost smooth.

Slide culture on 6% NaCl potato agar: No pseudomycelium

Assospore formation: Not found on 6% NaCl Gorodokowa agar and 6% NaCl "V-8" agar, observed over a three months-period.

Pellicle formation
- in NaCl-free koji extract at 20°C: no pellicle
- in 6% NaCl koji extract at 30°C: no pellicle
- in 18% NaCl koji extract at 30°C: no pellicle

Fermentation of sugars
- Fermented: glucose (week)
- Unfermented: galactose maltose, saccharose, lactose

Assimilation of sugars
- Assimilated: glucose
- Not assimilated: galactose, maltose saccharose, lactose

Assimilation of nitrate: Positive

Ethanol as sole source of carbon: No growth

Splitting of arbutin: Negative

yeast belongs to genus *Torulopsis* and resembles *Torulopsis glabrata* (*Anderson*) *Lodder et de Vries* or *Torulopsis inconspicua Lodder et Kreger-van Bij* in respect of its abilities to ferment and assimilate sugars. The present yeast, however, is distinguished from those species upon judgement in terms of nitrate assimilation. In addition to this difference, the present yeast showed obligate halophilic property at 30°C and facultative halophilism at 20°C as described above.

From these characteristics, the present author regarded the strain M 3 as a new species and named it *Torulopsis halonitratophila nov. sp.*

〔V〕 Influences of the Energy Sources and Sodium Chloride on Viability of *Torulopsis halonitratophila* at 20°C and 30°C.

The cells harvested from the fresh culture in 6% NaCl koji extract of *T. halonitratophila* were washed thoroughly and suspended in the following four types of media; ① pH 4.8 M/15 phosphate buffer; ② NaCl 6% in pH 4.8 M/15 phosphate buffer; ③ glucose 5% in pH 4.8 M/15 phosphate buffer; ④ NaCl 6% and glucose 5% in pH 4.8 M/15 phosphate buffer. The suspensions were incubated at either 20°C or 30°C. Viability of the yeast cells was determined by plating

M/15 phosphate buffer (pH 4.8)

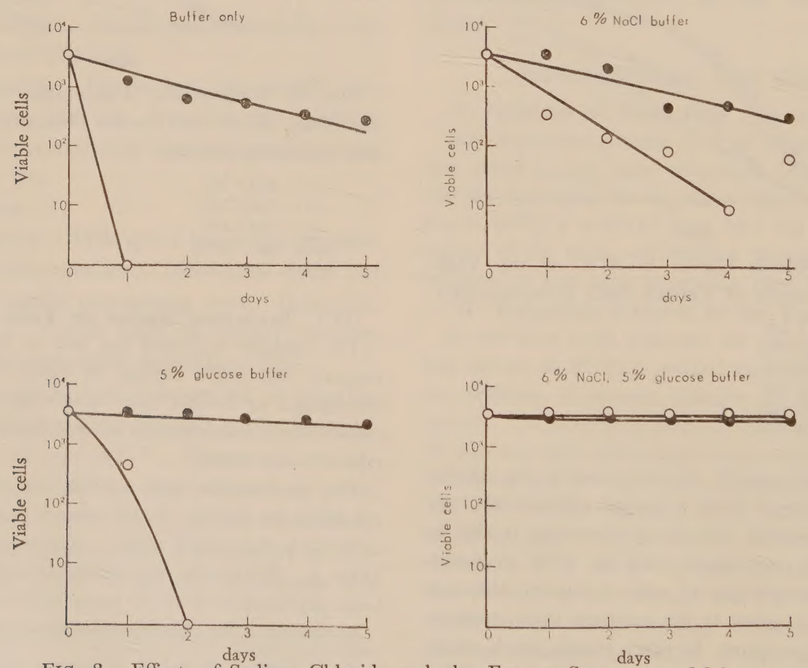

FIG. 3. Effects of Sodium Chloride and the Energy Sources on Maintenance of Cell-viability of *Torulopsis halonitratophila* at 20°C and 30°C. (1)

○: 30°C, ●: 20°C

aliquots on 6% NaCl koji agar. The plates were incubated at 30°C.

The results shown in Fig. 3, concerning the loss of viability at 30°C demonstrate that the addition of either 6% NaCl or 5% glucose had no protective effect on the cells. At the simultaneous presence of both glucose and NaCl in the suspension, the yeast maintained its viability. However, when incubation was carried out at 20°C, cell viability was fully maintained without the addition of NaCl and glucose.

In the following experiments, the cells were harvested from NaCl-free koji extract culture incubated at 20°C. Four types of cell suspension were made as described above and incubated at 30°C. As illustrated in Fig. 4, a similar phenomenon as shown in Fig. 3 was observed regardless the differences in the method of preculture.

These phenomena concerning cell viability were essentially identical with those of *Sacch. rouxii* which were reported in the previous paper[4]. However, the characteristic difference between these two strains was that the present isolated yeast failed to grow in the salt-free medium when it was tested at 30°C, which was the suitable temperature for growth of *Sacch. rouxii* as well as the majority of yeasts.

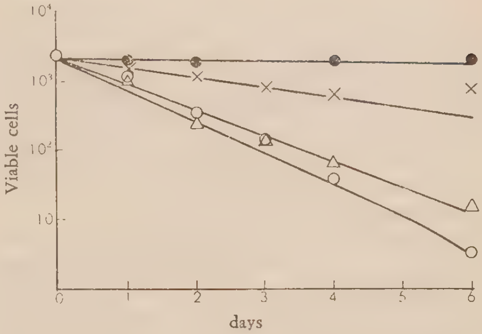

FIG. 4. Effects of Sodium Chloride and the Energy Source on Maintenance of Cell-viability of *Torulopsis halonitratophila* at 30°C. (2)

○ : M/15 phosphate buffer
× : 6% NaCl phosphate buffer
△ : 5% glucose phosphate buffer
● : 6% NaCl, 5% glucose phosphate buffer

[VI] Growth of *Torulopsis halonitratophila* in Various Hypertonic Media.

The methods of inoculation and cultivation were similar to those shown in [III]. As shown in Table II, *T. halonitratophila* exhibits appreciable growth at 30°C in various media containing $NaNO_3$, Na_2SO_4, KBr, KCl,

TABLE II. GROWTH OF *Torulopsis halonitratophila* IN VARIOUS HYPERTONIC MEDIA

Media			10 days	30 days
Basal medium*			−	−
〃	added with	0.5 Mol KBr	−	−
〃	〃	1 Mol KBr	⧺	⧺
〃	〃	0.5 Mol LiCl	−	−
〃	〃	1 Mol LiCl	−	−
〃	〃	1 Mol NaCl	⧻	⧻
〃	〃	0.5 Mol Na_2SO_4	−	⧻
〃	〃	1 Mol Na_2SO_4	⧺	⧻
〃	〃	1 Mol $NaNO_3$	⧺	⧻
〃	〃	2 Mol $NaNO_3$	⧻	⧻
〃	〃	1 Mol KCl	⧻	⧻
〃	〃	2 Mol KCl	⧻	⧻
〃	〃	1 Mol KNO_3	+	⧻
〃	〃	2 Mol KNO_3	⧻	⧻
〃	〃	0.3 Mol $CaCl_2 \cdot 2H_2O$	±	±
〃	〃	0.5 Mol $MgSO_4 \cdot 7H_2O$	−	−
〃	〃	1 Mol $MgSO_4 \cdot 7H_2O$	−	−
〃	〃	20% glucose	+	⧺
〃	〃	40% 〃	+	+
〃	〃	60% 〃	±	±

* Basal medium:

glucose	5%	KH_2PO_4	0.1%
$MgSO_4 \cdot 7H_2O$	0.5%	$CaCl_2 \cdot 2H_2O$	0.01%
casamino acid	0.4%	yeast ext.	0.1%

or KNO_3 instead of NaCl at high concentrations. The role of salts could be substituted with glucose at concentrations higher than 20 per cent.

〔VII〕 Standard description of *Torulopsis halonitratophila nov. sp.*

Growth in 6% NaCl koji extract: After kept 10 days at 30°C, cells are oval to ellipsoidal, $(2.5\sim3)\,\mu\times(4\sim5)\,\mu$. Only a sediment is formed.

Growth on 6% NaCl koji agar: After 2 weeks at 30°C, the streak culture is greyish brown, dull glistening, raised and almost smooth.

Slide culture on 6% NaCl potato agar: No pseudomycelium.

Ascospore formation: Not found on 6% NaCl Gorodokowa agar and 6% NaCl "V-8" agar, observed over a three month-period.

Fermentation: Glucose +(week) Maltose −
Galactose − Lactose −
Saccharose −

Sugar assimilation: Glucose + Maltose −
Galactose − Lactose −
Saccharose −

Assimilation of potassium nitrate: Positive
Ethanol as the sole source of carbon: No growth
Splitting of arbutin: Absent
Halophilic progerty: Obligate halophilic at 30°C. Facultative halophilic at 20°C.

DISCUSSION

Torulopsis inconspicua Lodder et Kreger-van Rij and *Torulopsis glabrate* (*Anderson*) *Lodder et de Vries*, which resembled the author's *Torulopsis halonitratophila*, were isolated from tannery liquid by Boidin and Abadie⁷⁾ and from concentrated orange juice by Recca and Mrak⁸⁾, respectively. These species have been found in concentrated solutions of salts or sugars. *T. halonitratophila* is distinguished from *T. inconspicua* and *T. glabrata* in respect of its ability to assimilate nitrate. It is of interest to note that *T. halonitratophila* does not form pellicles on any liquid media with or without NaCl in spite of its ability to assimilate nitrate. This property is considered to be a marked characteristic of most of salt-tolerant *Torulopsis* isolated from soy mash since many *Torulopsis sp.* showing such property besides *T. halonitratophila* have been isolated from soy mash by the present author⁶,⁹⁾

As described previously, *T. halonitratophila* shows obligate halophilic property at 30°C and is transformed into a faculative halophilic type when tested at 20°C. Such special halophilic property has so far, not been found in yeasts. In the case of a halophilic bacteria, *Halobacterium halobium*, Christian¹⁰⁾ has reported that the requirement of sodium chloride for growth becomes less exacting at relatively low temperature.

As a conclusion of these observations, it should be emphasized that cultural temperature is the most important factors for halophilism.

Acknowledgement The author wishes to express his sincerest thanks to Emeritus Prof. K. Sakaguchi of University of Tokyo for his kind guidance and encouragement throughout this work. Great indebtness is also acknowledged to Prof. T. Asai and Prof. Y. Sumiki for their valuable suggestions. The author's thanks are also due to Dr. M. Mogi, the director of Noda Institute for Scientific Research, for his kind encouragement in this work.

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7) J. Boidin and F. Abadie, *Bull. Soc. Mycol. France*, **70**, 353 (1954).

8) J. Recca and E. M. Mrak, *Food Technol.*, **6**, 450 (1952).

9) H. Onishi, This Bulletin, in press.

10) J.H.B. Christian, Dissertation, Cambridge (1956); cited from "Microbial Ecology" p. 102 (1957).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 2, p. 231~234, 1960]

Studies on the Formation of Glucosaminic Acid by *Acetobacter melanogenum* Beijerinck and *Pseudomonas fluorescens liquefaciens*

By Takeshi TAKAHASHI and Hisashi KAYAMORI

Department of Agricultural Chemistry, Faculty of Agriculture,
Tokyo University of Agriculture and Technology

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D-Glucosaminic acid has recently been found to be an oxidized product of D-glucosamine formed by *Ps. fluorescens*. It has been revealed that many strains of oxidative bacteria can oxidize glucosamine. The formation of glucosaminic acid has been recognized among a large number of strains of *Pseudomonas*, *Acetobacter* and *Gluconobacter*, by means of paper chromatography. Furthermore, one of these strains, *A. melanogenum* Beijerinck, oxidized glucosamine to glucosaminic acid with the theoretical consumption of oxygen as *Ps. fluorescens liquefaciens*. Glucosaminic acid was proved by isolation and identification by means of using resting cells.

The experiment of growth shows that *Ps. fluorescens liq.* could not secure any energy by means of the oxidation of glucosamine.

INTRODUCTION

According to studies on D-glucosamine metabolism with animal tissues and microorganisms, it is generally observed that glucosamine phosphorylated at the first step of metabolism and then deamination accompanied with the production of fructose-6-phosphate, which breakdown is followed by the pathway of sugar decomposition as usual.

Kusaka[1] found that in rat liver glucosamine changed to fructose without phosphorylation.

On the other hand, Imanaga[2] reported that *Pseudomonas fluorescens* oxidizes glucosamine to glucosaminic acid without phosphorylation.

The present authors presumed that many kinds of oxidative bacteria, especially, *Acetobacter* and *Gluconobacter*, can oxidize glucosamine to glucosaminic acid. It was found that glucosamine is oxidized to glucosaminic acid. It was found that glucosamine is oxidized to glucosaminic acid by *Acetobacter melanogenum* Beijerinck as well as by *Pseudomonas fluorescens liquefaciens.*

METHODS

The strains used in this study were *Acetobacter melanogenum* Beijerinck and *Pseudomonas fluoresens liquefaciens* and other strains kindly supplied by the Institute of Applied Microbiology, University of Tokyo. The above strains were preserved on a yeast extract slant containing glucose and a bouillon slant, respectively.

The following media were used for bacterial growth. A medium for *Acetobacter* consisting of 2% glucose, 0.5% glucosamine, 1% yeast extract, 1% calcium carbonate, and a different medium for *Pseudomonas* consisting of 0.5% glucosamine, 0.5% beef extract, 0.5% peptone. Glucosamine and other components of the medium were sterilized at 100°C for ten minutes and at 120°C for a quarter hour, respectively, and then mixed at inoculation added with calcium carbonate.

These organisms were incubated at 30°C for a day or two on a reciprocating shaker and collected by centrifugation. The cells were washed twice with 0.9% natrium chloride solution, resuspended and then used in the following experiments.

The oxygen uptake was measured by Warburg's manometer using resting cells. Most experiments were

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1) T. Kusaka, *J. Japan Biochem. Soc.*, **29**, 61 (1957).
2) Y. Imanaga, *J. Biochem.*, **44**, 819 (1957).

carried out with a medium of the following composition: Each vessel contained 0.5 ml of the cell suspension (about 2 mg as dry weight) and 1.0 ml of 1/5 M phosphate buffer (pH 7.0) in the main compartment, 50 μM of the substrate in the side arm and 0.2 ml of 20% potassium hydroxide in the center well. The total volume was 3.0 ml and the gas phase was filled up with air. The temperature was maintained at 30°C.

D-Glucosamine hydrochloride was prepared by hydrolyzing crab shells with hydrochloric acid and it was used after several recrystallizations.

N-Acetylglucosamine was synthesized from glucosamine hydrochloride[3].

Glucosaminic acid was prepared by oxidation of glucosamine with mercuric oxide[4].

The acetyl derivative of glucosaminic acid was formed by Neuberg's method[5].

Glucosamine was estimated by the method of Blix[6].

Ammonia contained in the reaction mixture was diffused into dil. sulfuric acid by means of Conway's microdiffusion apparatus, and then determined colorimetrically by means of Nessler's reagent[7].

RESULTS AND DISCUSSION

1. Oxidation of Glucosamine and Several Amino Compounds

In order to test whether or not the species of *Acetobacter*, *Gluconobacter* and *Pseudomonas* could generally oxidize amino sugars, experiments with resting cells were performed by employing Warburg's apparatus.

Table I shows that glucosamine is oxidized by many species of the above bacteria and N-acetylglucosamine is oxidized by *S. marcescens* and *E. coli* and not by other species. The oxidative activites of the above strains on glucosamine was less than on glucose except in cases of *S. marcescens* and *E. coli*. All strains used in this experiment could not oxidize glucosaminic acid and galactosamine.

A. melanogenum remarkably oxidized glucosamine by cells which were growing in the glucose medium similar to cells in the glucosamine medium, but oxidative activity on glucosamine of *Ps. fluorescens liq.* growing in glucose medium was very weak. The following glucose media were used. Thd medium for *Acetobacter* consisted of 2% glucose, 1% yeast extract and 1% calcium carbonate. Another medium for *Pseudomonas* consisted of 2% glucose, 0.2% ammonium sulfate, 0.1% potassium dihydrogen phosphate, 0.1% magnesium sulfate and 1% calcium carbonate.

TABLE I. OXIDATIVE ACTIVITIES ON SEVERAL SUBSTRATES BY RESTING CELLS OF SOME STRAINS

	O_2-Uptake μl/min./mg(cell dry *wt.*)		
	Glucose	Glucosamine	N-Acetyl-glucosamine
A. melanogenum	240	84	0
A. suboxydans	244	91	0
A. rancens	130	118	0
G. roseum	318	6	0
G. cerinus	474	50	0
Ps. fluorescens liq.	69	33	0
Ps. ovalis	23	9	0
S. marcescens	29	21	34
E. coli	48	44	51

The cells grown in glucosamine medium were used. Methods were described in the text. The results were corrected for the endogenous respiration.

Acetobacter and *Gluconobacter* may have a constitutive enzyme appropriate to oxidize glucosamine, because all strains of these species used in this study were able to oxidize glucosamine, in spite that they grew in glucose medium.

2. Total Oxygen Uptake

In this experiment an amount of 2 or 20 μM of the substrate was used and 2,4-dinitrophenol was added at the concentration of 5×10^{-5} M. Other conditions were in accordance with the above experiments.

By both species—*A. melanogenum* and *Ps. fluorescens liq.*, 0.5 μM of oxygen per μ mole of glucosamine was consumed respectively. This value agrees with the theoretical value in the case of formation of glucosaminic acid. *S. marcescens* and *E. coli* uptook $3\sim4$ μM of oxygen.

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[3] Y. Inouye, K. Onodera, S. Kitaoka and T. Kiri, *Bull. Inst. Chem. Research, Kyoto Univ.*, **33**, 270 (1955).

[4] H. Pringsheim and G. Ruschmann, *Ber.*, **48**, 681 (1915).

[5] C. Neuberg, *Ber.*, **35**, 4009 (1902).

[6] G. Blix, *Acta Chem. Scand.*, **2**, 467 (1948).

[7] E. K. Conway, "Microdiffusion Analysis and Volumetric Error", Crosby Lockwood, London (1950).

TABLE II. DEGRADATION OF GLUCOSAMINE BY RESTING CELLS

	Glucosamine consumed	NH_3 produced	$\frac{NH_3 \text{ produced}}{\text{Glucosamine consumed}}$	Paperchromatography Glucosamine	Paperchromatography Glucosaminic acid	Paperchromatography Other spot
	μM	μM	%	(R_F 0.43)	(R_F 0.34)	
A. melanogenum	187	3.1	1.7	trace	+	–
A. suboxydans	112	5.5	4.9	+	+	–
G. roseum	119	5.4	4.5	+	+	–
Ps. fluorescens liq.	81	trace	trace	+	+	–
Ps. ovalis	55	trace	trace	+	+	–
S. marcescens	85	26.6	31.3	+	–	–
E. coli	134	75.0	55.9	+	–	–

Resting cells (about 2 mg in dry wt.) were shaked with about 200 μM of glucosamine for five hours in 1/15 M phosphate buffer (pH 7.0). Ascending chromatography was used with following solvent system; *tert*-butanol-formic acid-water (7 : 1 : 3). The ninhydrin reagent was used for the coloring reaction of amino compounds.

3. Isolation and Identification of Glucosaminic Acid

Resting cells grown in glucosamine medium were suspended in 1/5 M phosphate buffer (pH 7.0) and incubate with glucosamine in a 1% concentration with shaking until reducing power disappeared. The cells were removed by centrifugation from the reaction mixture and the majority of phosphate salt was precipitated by adding barium hydroxide solution. Moreover, the remained cation was removed by the addition of Amberite IRC-50 (H-type). The reaction mixture was treated with active carbon and then concentrated in vacuo. By adding an equal volume of alcohol into the concentrated solution, the glucosaminic acid-like substance was crystallized and then recrystallized from water several times.

In this way, two glucosaminic acid-like crystal preparations were obtained from the broth of *A. melanogenum* and *Ps. fluorescens*.

According to paper chromatography of these preparations, their R_F value (0.34) agreed with that of the standard sample of glucosaminic acid. The conditions are described in Table II.

The acetylderivative of glucosaminic acid was prepared by Neuberg's method. The melting points of acetylderivatives of this preparation and the standard sample were both 123°C and the mixed sample of their preparations did not show a lower melting point.

The result of element analysis of the acetylderivative from *A. melanogenum* is shown below.

Anal. Found: C, 53.57: H, 5.27; N, 6.14%
Calcd. for $C_{10}H_{11}O_5N$: C, 53.31; H, 4.93; N, 6.22%

4. Degradation of Glucosamine and Production of Ammonia

Table II shows whether several organisms degradated glucosamine with or without deamination. *S. marcescens* and *E. coli* deaminated the majority of glucosamine consumed, while, in case of several species of *Acetobacter*, *Gluconobacter* and *Pseudomonas* only a little was deaminated.

The result of paper chromatography indicates that all strains of *Acetobacter*, *Gluconobacter* and *Pseudomonas* used here produce an amino compound that seems to be glucosaminic acid. Glucosaminic acid has recently been exhibited by Imanaga to be an oxidized product of glucosamine by *Ps. fluorescens*. Therefore it is apparent that formation of glucosaminic acid is a special character of this organism. These experiments, however, suggest that many strains of the oxidative bacteria except *S. marcescens* could generally oxidize glucosaminic acid.

5. Optimum pH

It has been observed that the optimum pH in the oxidation of glucose by *A. melanogenum* is in the acid side and its range is comparatively broad, whereas, in case of glucosamine it is limited in the range of 7.5~8.0 as shown in Fig. 1. The activity curve of *Ps. fluorescens* is not so sharp as that of *A. melanogenum.*

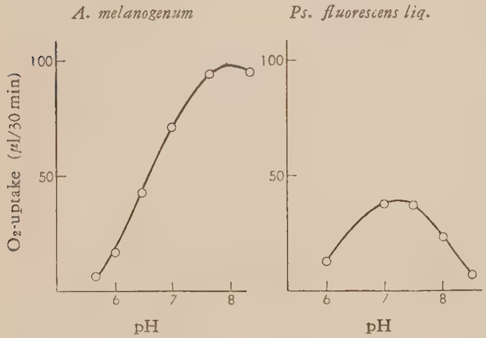

FIG. 1. Influence of pH on the Oxidation of Glucosamine

6. Influence of Glucosamine on the Growth of *Ps. fluorescens liq.*

In order to clarify the influence of glucosamine on the growth of *Ps. fluorescens liq.*, the following experiments were carried out. The used media were as follows: (1) As a negative control, 0.05% peptone and 0.05% beef extract, a growth-limiting quantity, were added to a basal salt solution; (2) as a positive control, 1% glucose was added to the negative control solution; and, (3) 1% or 5% glucosamine was added to the negative control solution. Monod's flasks were used for incubation and turbidity was measured nephelometrically.

The results are given in Fig. 2. Glucosamine remarkably inhibited the growth of *Ps. fluorescens* at the concentration of 5% or more. But, in the case of adding 1% glucosamine, the rate or growth was similar to the control.

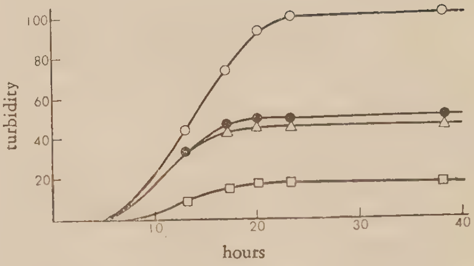

FIG. 2. Influence of Glucosamine on the Growth of *Ps. fluorescens liq.*

— ● — Basal medium, — ○ — 1% Glucose, — △ — 1% Glucosamine, — □ — 5% Glucosamine.

Campbell[8] has reported that *Ps. aeruginosa* is not able to secure energy during the oxidation steps, glucose→gluconate→2-ketogluconate.

Besides, Williams[9] recently reported that 2-deoxy-D-glucose is oxidized to 2-deoxy-D-gluconic acid by *Ps. aeruginosa* and does not serve as a source of carbon and energy.

Similary as in the above cases, this experiment shows that *Ps. fluorescens liq.* does not secure any energy by means of the oxidation of glucosamine.

Acknowledgement. The authors wish to express their sincere thanks to Dr. T. Asai and Dr. K. Aida for their kind advice and suggestions.

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8) J.J.R. Campbell, T. Ramakrishnan and A. G. Linnes, *Can. J. Microbiol.*, **2**, 304 (1956).

9) A. K. Williams and R. G. Eagon, *J. Bacterial.*, **77**, 173 (1959).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 235~242, 1960]

Enhancement of the Over-all Lethal Effect of Ionizing Radiations on Microorganisms by Sodium Chloride†

By Yoshishige OKAZAWA*, Mitsuo NAMIKI*, Satoru YAMASHITA*
and Akira MATSUYAMA**,*

The Institute of Physical and Chemical Research, Tokyo
***Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo*

Received August 14, 1959

Aiming at the enhancement of total lethal effect of radiations, chemical agent actions on irradiated cells were investigated. It was found that the sensitivity of irradiated cells to NaCl was markedly increased by radiation. In other words, the over-all lethal effect of radiations on microorganisms was enhanced by the pre- or post-treatment with NaCl. Some microbiological aspects of radiation enhancement of NaCl sensitivity were described in this paper, connecting with the possible mechanism. The fact that this phenomenon of radiation enhancement was observed in the case of the cells which gave the one-hit type survival curve suggests that radiation damage responsible for this phenomenon is not restricted to biological targets. Further discussion and experimental evidences will be presented in the subsequent paper.

INTRODUCTION

Radiation processing of foods as a newer attractive technology have been investigated by many workers and the potentiality of this technique was discussed[1~5]. Since 1957, the effects of ionizing radiations on foods, especially on the brewing products such as alcoholic beverages, miso and soy sauce which are widely used in this country, have been studied in our research group. It was demonstrated that deterioration in qualities of most brewing products occurred with sterilization doses of the oder of 10^5 to 10^6 r, althouth aging-like desirable effect of radiations on alcoholic beverages were found with relatively lower doses of 1 to 3×10^5 r.

Based on these experiments the most important problems in view of food irradiation preservation are considered as the following two, i. e. the enhancement of the total lethal effect on microorganisms in foods and the inhibition of the undesirable side reactions such as the off-flavor development and the color change, etc.

It has been elucidated that radiation effects can be modified by the treatment during or after irradiation. However, the enhancement of the total lethal effect of radiations by chemical agents has been little known except by oxygen and peroxides.

The study reported here was initiated with a view towards obtaining the highly over-all lethal effect of radiations on microorganisms, and this paper is concerned with the radiation enhancement of NaCl sensitivity. In the subsequent papers, data will be presented which implicate the possible mechanism of this radiation enhancement.

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† The abstract of this paper was published at the International Conference on the Preservation of Foods by Ionizing Radiations, at the Massachusetts Institute of Technology (U.S.A.) in July, 1959. And also this paper was partly presented in the Symposium on Effects of Radiations on Foods at the annual meeting of the Agricultural Chemical Society of Japan at the University of Tokyo in April, 1959.

1) B. E. Proctor and S. A. Goldblith, *Amer. J. of Public Health*, **47**, 439 (1957).

2) R. S. Hannan, Research on the Science and Technology of Food Preservation by Ionizing Radiations (1956).

3) B. H. Morgan, 2nd Intern. Conf. Peaceful Uses Atomic Energy, P/808 (1958).

4) M. Ingram, The paper presented at the Symposium on Effects of Radiations on Foods at the annual meeting of this society in April, 1959.

5) B. E. Proctor, ditto.

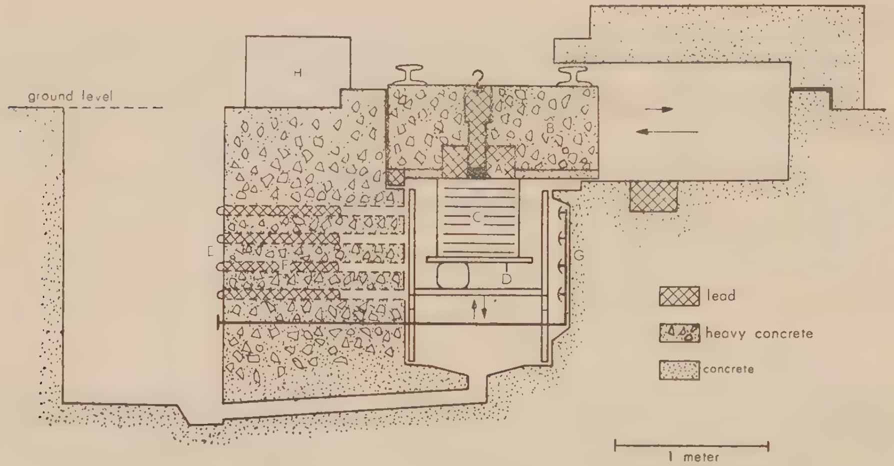

FIG. 1. Gamma Irradiation Apparatus for Biological Use

A, ^{60}Co source of coin type (270 curies)
B, source container (heavy concrete block being driven horizontally and automatically)
C, sample mounting being driven vertically and automatically
D, turn table
E, eyelet for observation
F, plug
G, mirror system
H, driving motor

MATERIALS AND METHODS

Microorganisms Three *Saccharomyces cerevisiae* strains designated 8256 (haploid), H-336 (diploid) and H-392 (tetraploid), *Saccharomyces sake* Kyokai No. 6, *Zygosaccharomyces soya* ZS-5, two *Escherichia coli* strains designated K-12 and 2-7, and *Bacillus subtilis* PCI-219, supplied by the Institute of Applied Microbiology, the University of Tokyo, were used in this work.

Gamma-ray irradiation The ^{60}Co source of 270 curies in the antibiotics laboratory of the Institute of Physical and Chemical Research[6,7] was used. This source consists of two pieces in coin type and is equipped in the center of the lower side surface of the heavy concrete block which has horizontal mobility (Fig. 1). The maximum dose rate was about 5×10^5 r/hr., and the dose rate usually used in this work was 2 to 10×10^4 r/hr.

Microorganisms were subcultured in the liquid media under shaking, washed two times by centrifugation and resuspended in the M/15 phosphate buffer at pH 6.0 (for yeasts) or 7.0 (for bacteria). Cell concentrations of suspensions were in the range of 10^3 to 10^7/ml. Total counts of cell suspensions were determinded using a hemocytometer prior to irradiation. Cell suspensions were exposed to gamma radiation at the room temperature, although they were cooled when above about 20°C.

Heat inactivation of cells Heating which gave the 1% survival was applied for the comparative study of irradiation and heat inactivation. In the water bath, cells (*Z. soya*) suspended in the phosphate buffer was heated at 48°C for 10 minutes and bacteria (*E. coli*) at 50°C for 15 minutes.

Experimental procedure For most of the experiments to be discussed in this work, the yeast cells except haploid cells were grown at 30°C on the nutrient YEPD agar (1% yeast extract, 4% peptone, 4% dextrose, 3% agar, pH 5.8). For the haploid yeast, Lindegren's medium[8] (pH 5.8~6.0) containing salts and vitamins was used. In some experiments yeast cells were inoculated into the liquid YEPD of the same composition (no agar) and subsequently the growth curves at 30°C were measured by means of viable counts.

Cell survival was taken as the ability of a cell to grow

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6) M. Namiki et al., *Reports of the Scientific Research Institute*, **33**, 81 (1957).

7) Y. Okazawa et al., Proc. 2nd Symposium on Atomic Energy of Japan, p. 289 (1958).

8) C. C. Lindegren and G. Lindegren, *Indian Phytopathology*, **4**, 11 (1951).

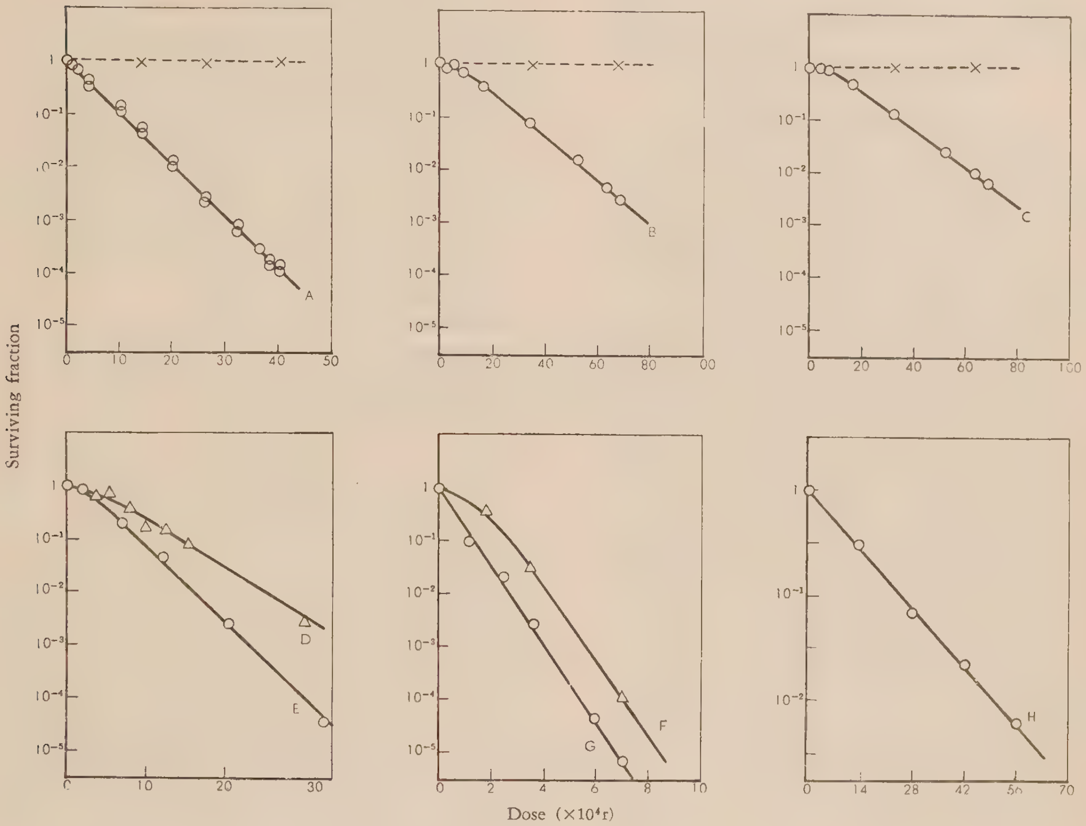

FIG. 2. Survival Curves

A, *S. cerevisiae* 8256 (haploid); B, *S. cerevisiae* H-336 (diploid); C, *S. cerevisiae* H-392 (tetraploid); D, *S. sake* Kyokai No. 6; E, *Z. soya* ZS-5; F, *E. coli* K 12; G, *E. coli* 2-7; H, *B. subtilis* PCI 219 spores.
Broken line shows viable counts of control suspension during irradiation

on nutrient agar into a visible colony after at least 5 days in a 30° incubator. Viable counting of yeast was carried out according to the ordinary plate method. Viable counts of *Escherichia coli* were determined by the capillary tube method[9,10]. The medium for this procedure was composed of 1% peptone, 1% meat extract, 0.5% sodium nitrate, 0.5% sodium chloride and 1.2% agar (pH 7.0). Colonies were scored after two day incubation at 37°C. However, the incubation was prolonged in the case of the medium containing inhibtory agents and it was acertained that no newer colony furthermore developed, similarly to the case of viable counting of yeasts. Viable counts of *Bacillus subtilis* spores were performed using the Petri dishes of 86 mm in diameter and nutrient agar (5% yeast extract, 0.3% peptone, 1% dextrose, 0.5% sodium chloride and 1.5% agar, pH 7.0). At first, 0.2 ml of spore suspension were placed on a center of the dish and then 6 ml of melted nutrient agar at about 45°C were poured onto the spore suspension, followed by homogeneous dispersion of spore in the nutrient agar by rotation. After coagulation of this agar layer containing spores, the second agar layer was formed on the first one by pouring about 5 ml of nutrient agar and subsequent rotation. Each of these two layers is about 1 mm in thickness. Plates were incubated at 37°C and the number of visible colonies was determined after two days, considering the note concerned with the case of inhibitory

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9) T. Yanagita, *J. Bact.*, **71**, 381 (1956).
10) A. Matsuyama, *J. Agr. Chem. Soc.*, **27**, 7 (1953).

environments as above mentioned. In the growth experiments, commercial NaCl (G.R. grade) was used and glass distilled water was employed.

RESULTS AND DISCUSSION

Survival curves Fig. 2, shows the survival curves of yeasts and bacteria exposed to irradiation of ^{60}Co gamma rays. Two types, which were exponential and sigmoidal, were found. For cells of haploid yeast and bacteria, single-hit type curves were observed over the wide range of surviving fractions, and there was no radioresistant tail on their survival curves. The viable counts of the unirradiated control suspensions were acertained to be constant throughout the periods of irradiation. The tetraploid yeast was more resistant than diploid yeast, although Mortimer[11] and Tobias et al.[12] reported polyploid yeasts (above diploid) was more radiosensitive than diploid yeast. This may be due to the fact that they were derived from different parent strains.

Growth of irradiated yeast in the presence of sodium chloride After gamma irradiation in NaCl-free phosphate buffer, growth curves of *Z. soya* were determined by means of viable counts (Fig. 3). The lag time was prolonged by irradiation even under the condition without sodium chloride. It is notable that the remarkable lethal effect of 13% sodium chloride on the irradiated yeast population was observed during the lag phase, whilst the same concentration of the salt was not so lethal for the unirradiated cells. There was no difference during the log and stationary phase of growth cycle of *Z. soya* in the media containing various concentrations of sodium chloride.

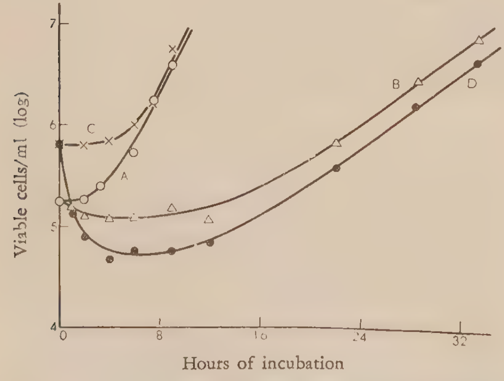

FIG. 3. Growth of *Z. soya* ZS-5 in Liquid Culture

A, control; B, unirradiated and added with 13% NaCl; C, irradiated by 1×15^5r,; D, irradiated by 1×10^5r and added with 13% NaCl; medium, YEPD agar

Surviving fractions on nutrient agar containing sodium chloride As might be expected from the above observation, it was also found that the viability of irradiated yeast cells was greatly depressed, even when cells were placed on a nutrient agar containing sodium chloride after irradiation in the NaCl-free environment. For example, *Z. soya* irradiated with a dose of 1.4×10^5 r (about 1% survival dose) could develop no visible colonies on the 14～15% saline medium which was not toxic to the normal control cells (Fig. 4). Such sensitizing effect of radiations (about 1% survival dose) on the salt action was also seen with *S. cerevisiae* 8256 (haploid), *S. cerevisiae* H-336, (diploid), *S. cerevisiae* H-392 (tetraploid), *S. cerevisiae* Kyokai No. 6, *E. coli* 2-7 and *E. coli* K 12 (Fig. 5). However, bacterial spores (*B. subtilis* PCI 219) were hardly sensitized by irradiation of gamma rays (Fig. 6).

It is important that these sensitizing effects of radiations are independent of the shape of the survival curves, i. e. whether it is exponential or sigmoidal. The radiation damage in living cells responsible for the above phenomenon may be not restricted to the alternative targets, because with single target inactivation by radiation, survivors should contain only undamadged targets from a view point of the target theory[13].

The experimental evidence for this view will be presented in a subsequent paper[14]. Furthermore, it is apparent that there is no correlation between the sensitizing effect by post-NaCl treatment and salt sensitivity of the unirradiated control cells. Relationship between radiation

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11) R. K. Mortimer, *Radiation Res.*, **9**. 312 (1958).

12) C. A. Tobias et al., 2nd Intern. Conf. Peaceful Uses Atomic Energy P/1844 (1958).

13) R. B. Uretz, *Radiation Res.*, **2**. 240 (1955).

14) Y. Okazawa et al., unpublished.

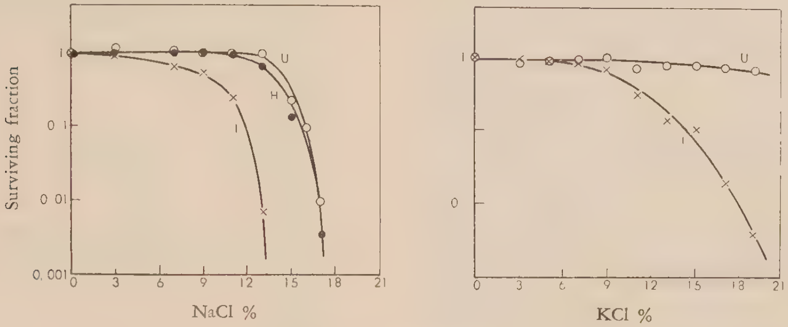

FIG. 4. NaCl and KCl Sensitivities of *Z. soya* ZS–5

U, unirradiated; I, irradiated (1.4×10 r); H, heated (48°, 10 min.); medium YEPD agar

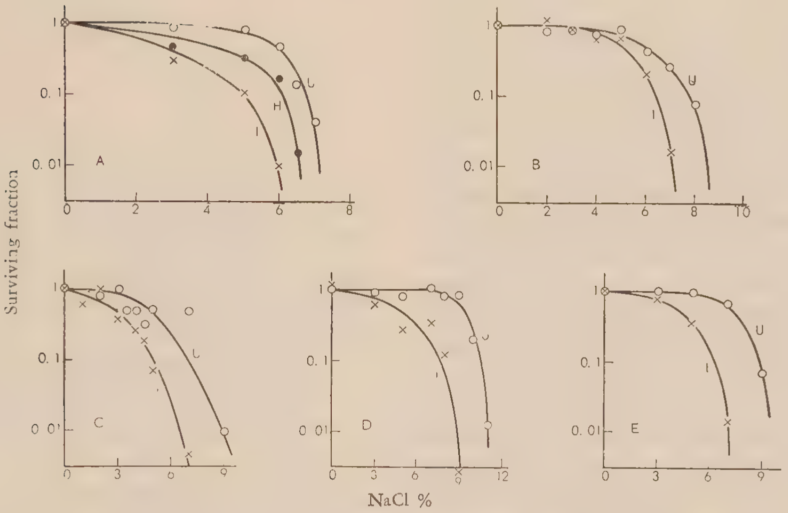

FIG. 5. NaCl Sensitivity of Microorganisms Subjected to Irradiated or Heat Inactivation

A, *E. coli* 2–7; B, *S. cerevisiae* 8256 (haploid); C, *S. cerevisiae* H-336 (diploid); D, *S. cerevisiae* H-392 (tetraploid); E, *S. sake* Kyokai No. 6; U, unirradiated; I, irradiated; H, heated

dose and enhancement of lethal effects by post-treatment with NaCl in a constant concentration is shown in Fig. 7. The enhancing effect increased proportionally (exponential type) or hyperbolically (sigmoidal type) with increasing radiation doses prior to the treatment with NaCl.

Comparison of sensitivity to salt action between irradiated and heat-inactivated cells Immediately after the gamma irradiation or heating (both of them gave the about 1% survival), cells were placed in the nutrient environment in the presence of the different concentrations of NaCl. The result, presented in Fig. 4 and Fig. 5.A, indicates that heated microorganisms are less sensitive to the toxic action of the post-treatment with NaCl than those irradiated with a dose equivalent to heating concerning the lethal effect.

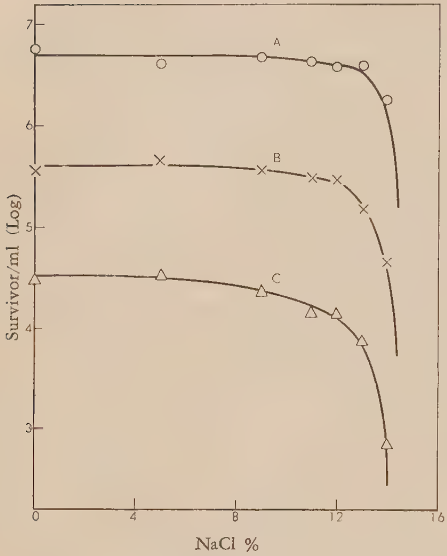

FIG. 6. NaCl Sensitivity of *B. subtilis* PCI 219 spores

A, control; B, 2.8×10^5r; C, 5.6×10^5r

Comparison of pre-treatments with irradiation and heat inactivation of cells in phosphate buffer Increase in sensitivity to the salt action was observed with heated cells in M/15 phosphate buffer as well as with irradiated cells. This fact and the above result illustrated in Fig. 8, suggest that injuries caused by heating are easily recovered, whilst radiation damage is hardly repaired. Salt action in phosphate buffer was not so toxic on unirradiated and irradiated cells in comparison with that in the nutrient environment. This is considered to be due to the mode of the salt action which is especially remarkable during the lag phase of growth cycle and so might associate with the cell division. At the higher temperature of incubation after irradiation, the salt action is a little more lethal.

Suvival curves on saline media As mentioned above, it was assumed that the enhancement of the lethal effect of radiation with sodium chloride is not restricted to targets, because even with single-hit type microbes this phenomenon was observed. Accordingly, from point of view that the shape of survival curve is a reflection of the radiation damage of targets in living

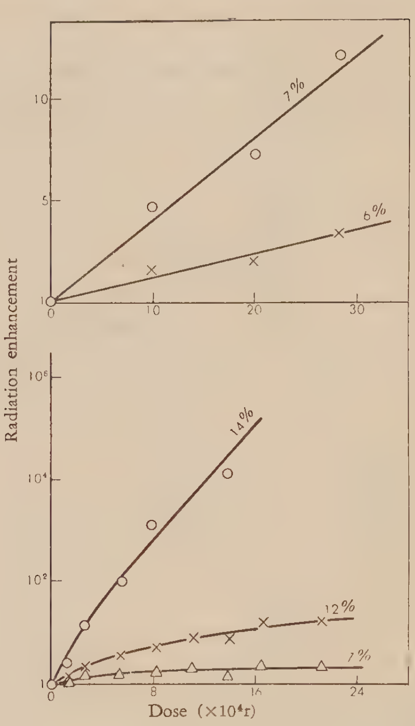

FIG. 7. Relationship between Doses and Radiation Enhancement of Lethal Effect of NaCl by Post-treatment on Nutrient Agar

A, *S. cerevisiae* 8256 (exponential survival curve); B, *Z. soya* ZS-5 (sigmoidal survival curve); Radiation enhancement is represented by decrease in surviving fraction from control (without NaCl); Per-centage shows the concentration of NaCl in nutrient agar (Based on data in Fig. 9)

cells, influence of salt action on the shape of the survival curve is of interest. The results in Fig. 9, show that the two-hit type survival curve of *Z. soya* was still sigmoidal on the nutrient agar containing 7 and 12% NaCl, but in the higher concentrations of NaCl the curve approach to the exponential type. This fact supports the above view that non-selective damages within the cells by radiations occur in the enhancing process of the lethal effect by post-NaCl treatment.

Action of the salt during irradiation on cells in phosphate buffer The data of Fig. 10, show a set of curves indicating the NaCl action during and after irradiation on cells. With the lower concentrations the enhancement of lethal effect of radiation by th salt action during irradiation was remarkable than with the same concentration of the salt added after irradiation. Based on the results in Fig. 10, if the periods for post-treatment is not so long, it is considered that the total lethal action of NaCl on irradiated resting cells are attributable mainly to that during irradiation and partly to that after irradiation, because surviving farctions decreased by the irradiation in the presence of NaCl more significantly than by the post-treatment with the salt. Comparing the results in Figs. 8 and 11, such tendency was observed in the case of irradiation of yeast. This fact suggests that long-life species which might be produced in buffer by radiations do not play any important role in the radiation enhancement of cell sensitivity to the salt actions. Such enhancing effect by higher concentrations of the salt during irradiation, however, is not so remarkably increased and so the curve showing the effect of concentrations of NaCl has the radioresistant tail. As the possible explanation for this radioresistant tail, two assumptions are at least probable: i.e. the restoration from radiation damage and the involvement of radioresistant fraction

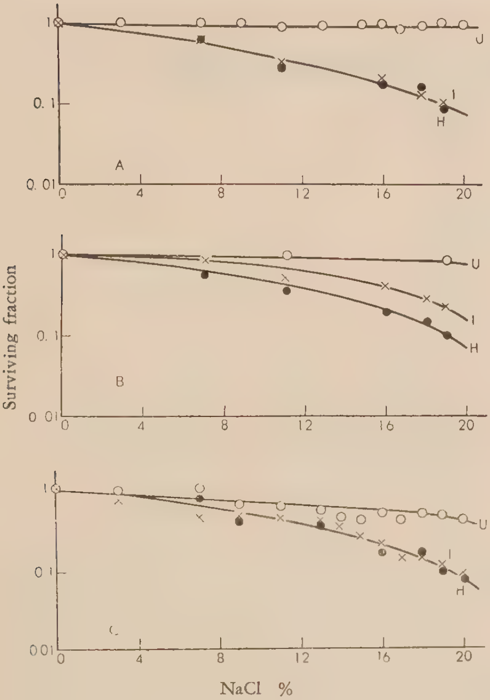

FIG. 8. Sensitivity of Irradiated and Heated Cells (*Z. soya* ZS-5) to NaCl Action

U, untreated; I, irradiated by 1.4×10^5r; H, heated at 48° for 10 minutes. Treatment with NaCl after irradiation; A, 4°, 18 hours; B, 4°, 4 hours; C, 30°, 4 hours

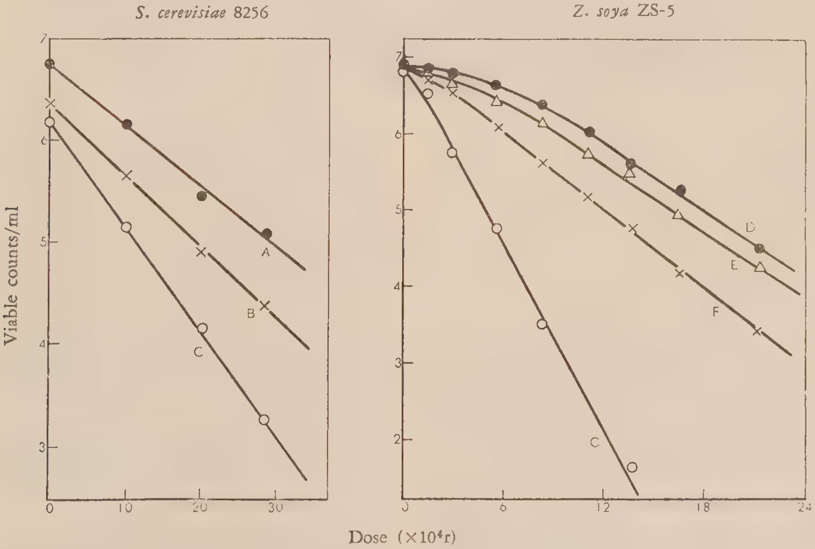

FIG. 9. Survival Curves of Yeasts on Saline Nutrient Agar

NaCl %: A, 0; B, 6; C, 7; D, 0; E, 7; F, 12; G, 14.

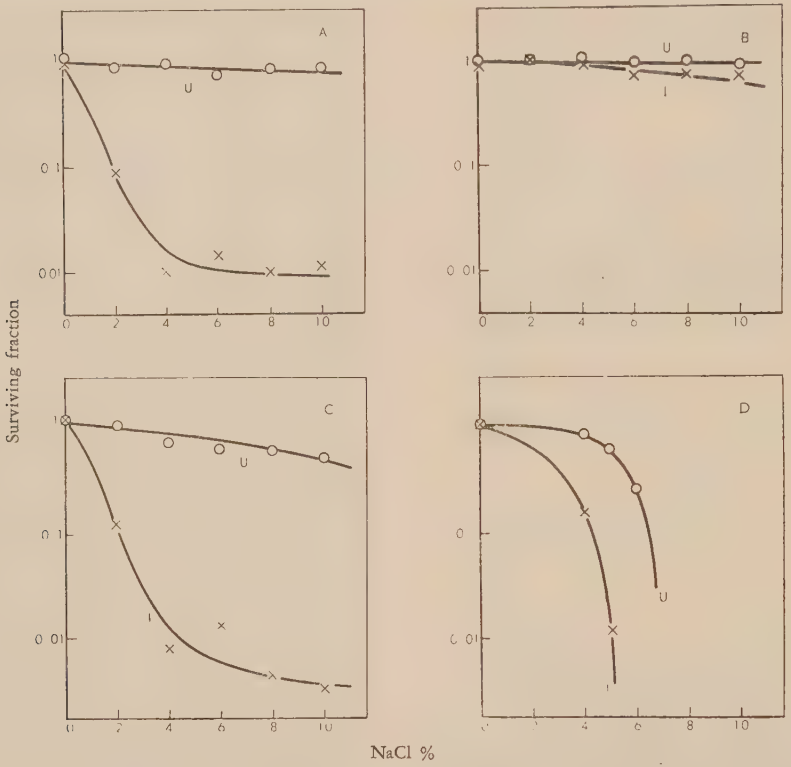

FIG. 10. NaCl Action on Bacterial Cells (*E. coli* K 12) during and after Irradiation

A, irradiated in NaCl phosphate buffer (pH 7.0) by 4×10^4r (during 1.5 hours); B, irradiated in NaCl free phosphate buffer (pH 7.0) by 4×10^4r and then treated with NaCl for 2 hours; C, irradiated in NaCl phosphate buffer (pH 7.0) by 4×10^4r and standing for 2 hours after irradiation similarly to B (A, B and C were grown on salt-free nutrient agar); D, irradiated in salt-free phosphate buffer and grown on saline nutrient agar immediately after irradiation by 4×10^4r.

In each case, U and I represent unirradiated and irradiated cells, respectively.

in the cell population. The further discussion of this problem will be presented in the subsequent paper.

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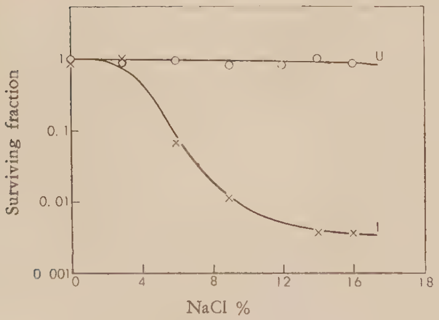


FIG. 11. NaCl Action on Yeast Cells (*Z. soya* ZS-5) during irradiation

I, irradiated in NaCl phosphate buffer by 1.4×10^5r and then grown on saline nutrient agar at 30°; U, control.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 243~248, 1960]

Metabolism of Aromatic Compounds by Microbes

Part VII. Gentisic Acid Oxidase

By Shin-ich SUGIYAMA, Keiji YANO, Kazuo KOMAGATA
and Kei ARIMA

Department of Agricultrual Chemistry, Faculty of Agriculture, University of Tokyo

Received July 11, 1959

A new enzyme which catalyzes an oxidative breakdown of gentisic acid with rupturing of the benzene ring was extracted from the *m*-hydroxybenzoic acid induced cells of *Pseudomonas ovalis* S-5 by several methods and named "Gentisic acid oxidase".

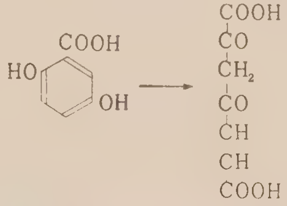
The enzyme was partially purified by various methods such as precipitation with ammonium sulfate, and ferrous ion was proved necessary for the activity of this enzyme.

Since, pyrocatechase was first discovered by M. Suda et al., in 1950, several other benzene ring splitting enzymes such as homogentisic acid oxidase[1,2], protocatechuic acid oxidase[3], 3-hydroxyanthranilic acid oxidase[4] have been reported. The present authors have investigated many bacteria isolated from soil and sewages which metabolize aromatic compounds, and in the bacterial metabolism of *m*-hydroxybenzoic acid (*m*-HBA), have demonstrated two pathways, the one the path *via* gentisic acid (GA) and the other *via* protocatechuic acid (PCA) from which it was found that the two pathways do not exist together in any bacterial strain.

Pseudomonas ovalis S-5 is one of the strains which oxidatively metabolize *m*-HBA *via* GA, accompanied by a cleavage of the benzene ring of GA. The authors have reported on the factors influencing enzyme formation in sequential induction methods[5,6].

The cell-free enzyme preparation which can oxidize GA was extracted from *m*-HBA induced cells of *Pseudomonas ovalis* S-5. This enzyme is a new one and requires ferrous ion as a sole cofactor and is named "Gentisic acid oxidase". As reported in the following paper, this β-diketo-dicarboxylic acid was isolated in a crystalline form from the enzyme reaction mixture and was identified as fumarylpyruvic acid. Further the initial intermediate maleylpyruvic acid has been also recognized spectrophotometrically by using a purified enzyme preparation[7].

The present paper is concerned with the extraction, partial purification and certain properties of gentisic acid oxidase, which catalyzes the following reaction:



MATERIALS AND METHODS

Biological: *Pseudomonas ovalis* S-5. The cells were grown in 100 ml of bouillon-peptone broth in a 500 ml Sakaguchi flask. Culture was continued for 18 hrs at 30°C with constant shaking. The cells were harvested by centrifugation, washed once with dist. water, and induced in M/15 phosphate buffer, pH 7.2 (wet cells 10 g/100 ml) by M/100 *m*-HBA as an inducer[5,6].

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[1] M. Suda and Y. Takeda, *J. Biochem. Japan*, **37**, 375 (1950).

[2] D. L. Crandall, *J. Biol. Chem.*, **212**, 565 (1955).

[3] R. Y. Stanier and J. L. Ingraham, *J. Biol. Chem.*, **210**, 799 (1954).

[4] R. W. Schayer and L. M. Henderson, *J. Biol. Chem.*, **195**, 657 (1952).

[5] K. Arima, K. Komagata, S. Sugiyama, K. Kazama and K. Yano, This Bulletin, **19**, 51 (1954).

[6] K. Arima, K. Komagata, K. Kazama, S. Sugiyama and K. Yano, This Bulletin, **19**, 61 (1954).

[7] S. Sugiyama, K. Yano, H. Tanaka and K. Arima, This Bulletin, **24**, 255 (1960)

Determination and Chemicals: Enzymatic activity was determined by oxygen uptake using Warburg's manometer.

Contents of the Warburg vessel

Main compartment	Enzyme solution	0.5 ml
	M/15 phosphate buffer pH 7.2	0.4
	Ferrous ion solution*	0.1
Center well	10 per cent KOH	0.1
Side arm	10^{-2} M sodium gentisate	0.3~0.5

* A mixture of ferrous sulfate and ascorbic acid (prepared at the final concentration of 10^{-3} M)

Specific activity is shown as Qo_2 (protein). Protein was determined by the micro-Kjeldahl method and by Folin-Ciocalteu method after precipitation by 10 per cent trichloracetic acid. GA used as the substrate was recrystallized before use.

Preparation crude enzyme extracts: Since the induced cells were completely inactivated by drying with cold acetone and lost their activity by vacuum dry treatment, the cell-free extract was obtained directly from the intact induce cells as shown in Table I.

TABLE I. METHODS FOR PREPARATING CELL-FREE EXTRACTS

		Oxygen uptake
A)	From wet cells	
	1) Grinding with Alumina	⧺
	2) Grinding with Pyrex glass powder	⧺
	3) Grinding by Potter's homogenizer	+
	4) Ultra-sonic Vibration*	+
B)	From dry cells	
	1) Grinding with Alumina	+
	2) Grinding with Pyrex glass powder	+

* Vibrating condition of apparatus was 560 kc, 800 W

Although the enzyme activity could be demonstrated by each treatment, a considerable loss was always recognized except in case of grinding the intact cells with alumina. Extraction by sonic vibration was effective for large scale treatment, however, a considerable decrease of activity which might be caused by oxidation during the treatment was observed. Therefore, throughout this experiment the enzyme was extracted by the method of grinding with alumina.

The wet, packed induced cells were ground by hand for 30 min. with the same weight of levigated alumina. All preparative procedures were conducted from 0 to 4°C. The mixture was suspended in 10 ml of M/15 phosphate buffer (pH 7.4) per 10 g of wet cells.

The bulk of alumina was removed by low speed centrifugation and then the particulate fraction of the extracts was sedimented by refrigerating centrifugation for 30 min. at 10,000 × g and discarded. A clear supernatant solution was obtained.

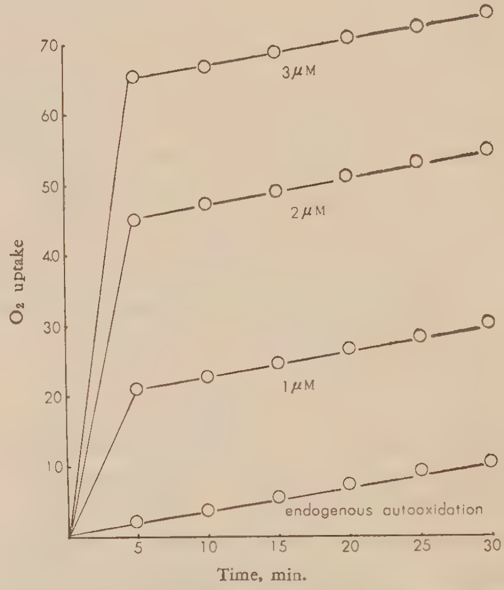

FIG. 1. O_2 Uptake for Gentisic Acid Oxidation by Crude Enzyme Preparation

Several properties of crude extracts: Fig. 1 shows oxygen uptakes required for GA oxidation by crude extracts. One mole of oxygen was consumed per mole of GA. The oxidative activity of this enzyme preparation on PCA was demonstrated. However, *m*-HBA was not oxidized at all by this enzyme preparation. This preparation was stable at −20°C for one year, therefore, the enzyme solution were stored in a frozen state. Considerable losses of activity resulted after dialysis of the enzyme solution against dist. water or M/30 phosphate buffer pH 7.4 with agitation for 18 hrs, and almost complete losses of activity were shown against the stream water. From the above experiments it was suggested that the enzyme required some substance as a co-factor, such as metal ion which was rapidly removed from the apoenzyme. Table II shows the effect of various metal ions for the dialyzed enzyme. By the addition of 10^{-3} M ferrous ion, the inactivated preparation was almost restored to its original activity, though not completely, and was also not reactivated with ferric ion. When ascorbic acid was added with ferrous ion in order to maintain the level of ferrous ion, the effect

TABLE II. THE EFFECT OF METALIONS TO DIALYZED PREPARATION

Metal ions	Final concentration	O_2 uptake in 30 min.
Fe^{++}	10^{-3} M	47.63
Fe^{+++}	10^{-3}	5.99
Fe^{++}+V.C	10^{-3}	62.31
Zn^{++}	10^{-3}	0
Mg^{++}	10^{-3}	0
Mo(Na_2MoO_4)	10^{-3}	0
Mn^{++}	10^{-3}	0
Co^{++}	10^{-3}	0
Cu^{++}	10^{-3}	0
Non add. (dialyzate)		0
Nondialysis preparation		63.75

Each ion adds form as sulfate except to Mo^{++}.

for the reactivation was greater than when they were used alone. Other metal ions can not replace ferrous ion as a co-factor.

The effect of other reducing agents such as glutathione, cystein were shown in Fig. 2, where glutathione (10^{-3} M) gave the same effect as ascorbic acid. The role of ferrous ion on enzyme activity is described in a later paper⁸⁾.

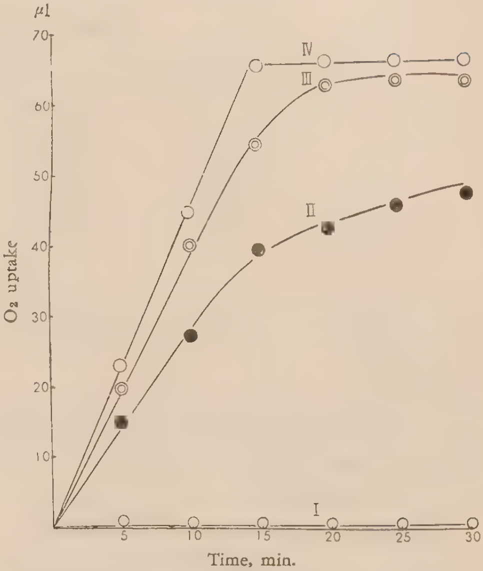

FIG. 2. The Effect of Glutathione or Cystein for Maintain the Level of Ferrous Ion

I Dialyzed preparation: incubation of enzyme and substrate only
II With ferrous ion 2×10^{-3}M
III With ferrous ion and glutathione or cystein
IV Non-dialyzed preparation
The concentration of substrate was 3 μM in each case.

As preliminary experiments for the enzyme purification, the following tests were carried out.

Heat treatment: Crude extracts adjusted at several pH values (5.4, 6.0, 7.2, 8.0, 9.2) were heated with stirring in a water bath at several temperature levels (50, 70, 100°C) for 3 to 5 minutes. Then they were immediately cooled by ice, and the resulted precipitate was removed by centrifugation and discarded. Activity of each supernatant was determined as shown in Fig. 3; the enzyme remained its activity at 50° for 3 min, but specific activity was decreased considerably. In respect to the relation between stability, this enzyme was most stable at pH 7.2 (see Fig. 3).

Adsorption by adsorbants: Suitable conditions were not obtained, because the crude extracts contained plenty of nucleic acids. The following adsorbants were tested for their adsorption effect. As enzyme activity is prominently decreased in the acid side any adsorption procedure was carried out in neutral and the slight alkaline side.

Calcium phsophate gel: The technic employed here was simply to mix the adsorbent and solution together, separate the adsorbent, and elute the enzyme and activity of supernatant and eluted solution were determined⁹⁾. As described in the later report⁸⁾ the adsorption effect of this gel was influenced by the existence of the phosphate ions in the enzyme preparation. Though the enzyme preparation extracted with phosphate buffer was not adsorpted with this gel in neutral pH ranges, the preparation extracted with trisaminomethane-HCl buffer was adsorpted strengthly, even if when they were treated at neutral pH ranges.

(2) **Hyflosupercel** Either in neutral pH or the acid side, the enzyme was not adsorpted by this adsorbent.

(3) **Active alumina** This was not a suitable adsorbent, as the pH value went up to 10.0 during the course of adsorption treatment.

Centrifugation: Crude extracts prepared by refrigerated centrifugation at 10,000×g for 30 min, and the supernatants prepared by ultracentrifugation at 144,7000×g for 30 min. maintained the whole of their activities.

From the results of the above experiments, purification of the enzyme was carried out.

To simplify the explanation, procedures of the enzyme purification is illustrated by the following scheme.

All the preparative procedures were carried out at 0

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8) S. Sugiyama, K. Yano and K. Arima, This Bulletin, **24**, 249 (1960)

9) D. Keilin and E. F. Hartree, *Biochem. J.*, **49**, 88 (1951).

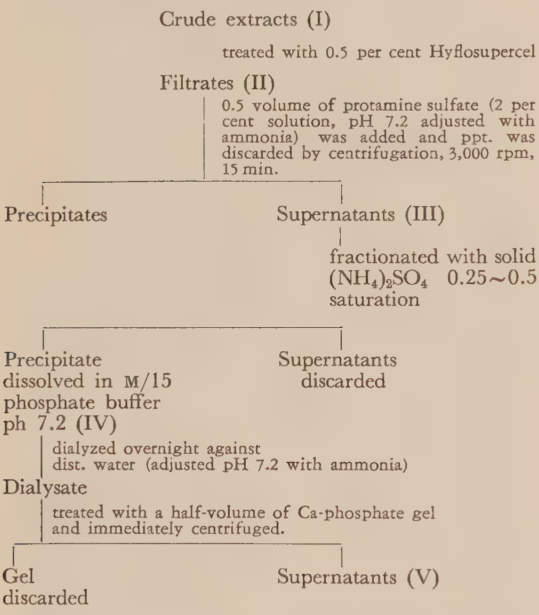
TABLE III. PARTIAL PURIFICATION OF GENTISIC ACID OXIDASE

Fraction	Volume	Protein (mg)		Specific activity	Yield
	ml	per ml	total	Q_{O_2} protein	%
I	110	29.2	3,212	123	100
II	105	21.7	2,281	165	95.3
III	147	13.5	1,981	181	91.3
IV	20	10.2	204	1,125	57.9
V	20	9.7	193	1,150	56.2

~4°C. Throughout the course of purification, ferrous ion and ascorbic acid were added to each fraction as an enzyme protector.

Thus, the amber-colored and transparent preparation Fraction V was obtained. Its specific activity was calculated to be Q_{O_2} (protein)=1150 (Table III), which is 8 times that of Fraction I.

DIAGRAM OF PURIFICATION PROCEDURE


Crude extracts (I)
treated with 0.5 per cent Hyflosupercel
Filtrates (II)
0.5 volume of protamine sulfate (2 per cent solution, pH 7.2 adjusted with ammonia) was added and ppt. was discarded by centrifugation, 3,000 rpm, 15 min.
Precipitates
Supernatants (III)
fractionated with solid $(NH_4)_2SO_4$ 0.25~0.5 saturation
Precipitate dissolved in M/15 phosphate buffer ph 7.2 (IV)
Supernatants discarded
dialyzed overnight against dist. water (adjusted pH 7.2 with ammonia)
Dialysate
treated with a half-volume of Ca-phosphate gel and immediately centrifuged.
Gel discarded
Supernatants (V)

Properties of the partially purified enzyme (Fraction V): The oxygen uptake for the oxidation of GA was the same as that of crude extracts. One mole of oxygen per one mole of sodium GA was consumed and endogenous autooxidation was not detected. Since CO_2 was not evolved in the course of these reactions, as seen in Table IV, it is suggested that the oxidative decarboxylation had not occurred and a seven carbon intermediate is produced accompanied with benzene ring fission.

TABLE IV. CO_2 EVOLUTION BY OXIDATION OF GENTISIC ACID

		Sample	non substrate
Total O_2 uptake	μl	56.60	+1.91
O_2 consumed calculated	mm	−22	+3
Observed pressure change due to add H_2SO_4 at start	mm	+13.1	+8.0
CO_2 evolution from phosphate buffer	μl	26.7	+17.45
CO_2 evolution calculated	mm	13.3	8.3
Observed pressure change due to add H_2SO_4 after 1 hr.	mm	−12	+9
Net pressure change due to oxidation calculated	mm	−3.3	−3.29
CO_2 produced by oxidation	μl	−6.612	−6.927
Net CO_2 produced by oxidation	μl	0.32	

Substrate concentration was 3 μM

TABLE V. CO_2 PRODUCTION IN $NaHCO_3$ SOLUTION WARBURG'S VESSEL CONTENT

Min.	Control O_2 uptake	CO_2 evolution in M/25 $NaHCO_3$
1	5.88	1.12
2	10.58	3.70
3	16.46	8.94
4	21.16	14.21
5	25.87	19.49
8	39.98	33.95
10	47.03	40.49
15	57.62	52.37
20	57.62	52.37
30	58.80	54.96

Control Usual Content

M/25 $NaHCO_8$ solution	1.75 ml
Enzyme solution	0.25 ml
15% CO_2 in air.	gas phase
M/100 gentisate	0.3 ml

Table V shows carbon dioxide evolution during GA oxidation which was carried out in the CO_2-$NaHCO_3$

buffer. In this condition one mole of CO_2 per mole of GA oxidation was evolved. This means that one mole of carboxyl radical is produced in the reaction mixture by enzymic oxidation of GA. As described in the previous report, the incubation mixture of induced cells with GA gave positive Rothera's reaction and produced 2,4-dinitrophenylhydrazone. Furthermore, the reaction mixture of this partially purified enzyme reacted specifically with *o*-phenylenediamine. These facts supported the assumption that the direct product of GA oxidation accompanying the benzene ring cleavage is seven carbon β-diketodicarboxylic acid. As described in the appendix, the authors succeeded in the isolation and identification of fumarlypyruvic acid, and spectrophotometrically recognized maleylpyruvic acid which is the initial intermediate of GA metabolism of this *Pseudomonas*. The optimum substrate concentration is from 8 to 10 μM of GA, Km is 2×10^{-3} M.

Optimum pH: As seen in Fig. 4 the pH optimum of this enzyme preparation lies at pH 8.0. The addition of ferrous ion and ascorbic acid can recover enzyme activity at a low pH adjusted with 1 N acetic acid, similar to the case of homogentisic acid oxidase. From this, it is suggested that ferrous ion plays a role in stabilization of the enzyme.

Specificity: Several phenolic compounds were tested as substrates. As shown in Table VI, none of the following compounds catechol, homogentisic acid, 2,3-dihydroxybenzoic acid, and β-resorcilic acid were oxidized. Hence this enzyme differs distinctly from pyrocatechase and homogentisic acid oxidase. Though gentisic acid attacked as the substrate is out of question, it is a remarkable fact that protocatechuic acid is known to be attacked. From this phenomena, two possibilities are considered: a) the gentisic acid oxidase has also oxidative activity on protocatechuic acid, b) protocatechuic acid oxidase contaminates in this preparation. According to other experiments as will be described in the following paper[8], the cell free extracts from non-induced cells also attack PCA. Thus, it is clear that protocate-

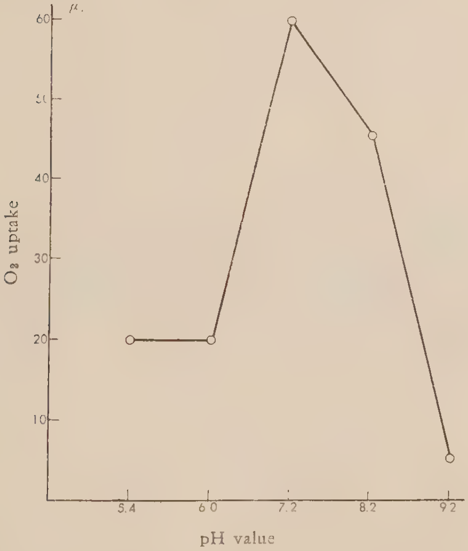


FIG. 3. 50°C Heat Treatment at several pH Range for 5 min. on Crude Extracts

Substrates concentration .... 3 μM

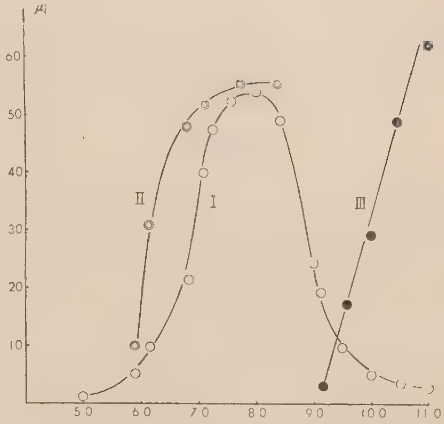


FIG. 4. The Effects of pH on Gentisic Acid Oxidase

I; pH-activity curve, incuvation of enzyme and substrate only
II; With ferrous ion 2×10^{-8} M and ascorbic acid
III; G.A autooxidation

Phosphate buffer was use from pH 5.1 to 7.5 and Tris buffer from pH 8.0 to 9.0, and carbonate-bicarbonate buffer from pH 9.2 to 10.7

TABLE VI. SUBSTRATE SPECIFICITY OF PARTIALLY PURIFIED SAMPLE

o-Hydroxybenzoic acid	0 ml
m-Hydroxybenzoic acid	0
p-Hydroxybenzoic acid	0
Gentisic acid	67.02
Protocatechuic acid	61.00
Homogentisic acid	0
Catechol	0
β-Resorcilic acid	0
2,3-Dihydroxybenzoic acid	0

O_2 uptake in 30 minutes
Concentration of each substrates were 3 μM

chuic acid oxidase contaminates in this preparation and this strain has protocatechuic acid oxidase as the constitutive enzyme. The present authors have succeeded to separate both enzyme each other.

DISCUSSION

Gentisic acid oxidase is a new oxidase and requires ferrous ion as its sole co-factor similar to the other benzene ring splitting enzymes. It seems to be very interesting that these benzene ring splitting enzymes require ferrous ion, though a small difference exists in the liberation of ferrous ion from the apoprotein. In the case of gentisic acid oxidase, ferrous ion is liberated very easily by dialysis and salting out by ammonium sulfate. Furthermore since the use of phosphate buffer in extracting the enzyme from disrupted cells is unfavourable, because the precipitated, ferrous ion and phosphate ion complex, occurred and pH value decreased to some degree, we examined the application of trishydroxyaminomethane-HCl buffer for the purpose of extraction.

In order to obtain the crude extracts, disruption of the cells by application of ultrasonic vibration seemed to be a convenient method for large scale treatment. This enzyme, however, was labile by heat treatment and required ferrous ion as a co-factor which was easily oxidized, so that the extracts obtained by sonic disintegration lost their activity considerably. Therefore, the extracts were obtained by grinding with levigated alumina, with caution on cooling treatment and maintenance of reductive state during sonic treatment.

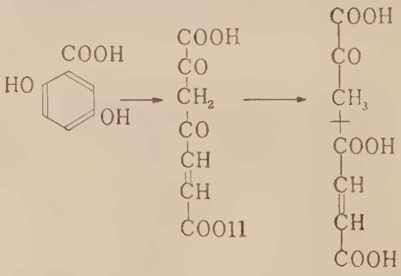
It should perhaps be possible to obtain the extracts without a loss of the activity caused by sonic treatment, paying attention to keep the content cooled and giving heed to the reductive agent.

SUMMARY

(1) Cell-free enzyme preparation which catalyzes an oxidative breakdown of gentisic acid with rupturing of benzene ring was obtained from *m*-hydroxybenzoic acid induced cells of *Pseudomonas ovalis* S-5. This new benzene ring splitting enzyme was named "Gentisic acid oxidase".

(2) Comparisons of various methods to obtain a cell free enzyme preparation were made. The enzyme was partially purified, and ferrous ion was proved necessary, for the activity of this enzyme. Further, some other properties of the gentisic acid oxidase were also investigated.

(3) From the various evidences obtained by manometrical experiments and chemical reaction of the product of this enzyme, the oxidative pathway of gentisic acid by *Ps. ovalis* was presumed to be as follows.



Acknowledgement The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continuous guidance throughout this work.

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Metabolism of Aromatic Compounds by Microbes

Part VIII. Further Studies of Centisic Acid Oxidase

By Shin-ich SUGIYAMA, Keiji YANO and Kei ARIMA

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

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A highly purified gentisic acid oxidase from *Pseudomonas ovalis* S-5 was obtained and purification procedures are described in this report. The specific activity increased up to twenty fold. This enzyme specifically oxidizes gentisic acid with a comsumption of 1 M of oxygen per 1 M of the substrate yielding fumarylpyruvic acid. Properties of this enzyme were extensively investigated. This enzyme required ferrous ion as sole cofactor. Several metal chelating inhibitors, analogue inhibitors and the reactivators as ascorbic acid, effect of preincubation and possible participation of SH-radical in the enzyme-substrate combination etc. were studied. And comparison of all the known five benzene ring splitting enzymes were made.

In the previous report[1], the authors obtained cell-free enzyme preparation from *Pseudomonas ovalis* S-5 which catalyzed the oxidative breakdown of gentisic acid (GA), and named it "gentisic acid oxidase." This enzyme, which required ferrous ion as a sole cofactor, was a new benzene ring splitting enzyme. The characteristics of gentisic acid oxidase show the similarity to the other enzymes as pyrocatechase, homogentisic acid oxidase.

The partially purified preparation comsumed 1 M of oxygen per 1 M of GA and the carbon dioxide evolution was not found. In the reaction mixture, an unknown substance which was colored specifically by *o*-phenylenediamine was accumulated. The authors presumed that this substance was the initial intermediate after the oxidative ring cleavage of GA, which might be maleylpyruvate or fumarylpyruvate.

As described in the later paper, we have recently succeeded in isolating one of the presumed intermediate in crystalline form[2]. Present communication is concerned with further purification and properties of this enzyme.

EXPERIMENTAL AND RESULTS

The biological source, the cultural conditions, the methods of induction of the enzyme, the method of extraction of the cell-free enzyme, and the method of determination of the enzyme activity were the same as described in the previous report. The determination of protein war conducted by Folin-Ciocalteu methods[3] after precipitation by 10 per cent trichloroacetic acid. In the previous report, M/15 phosphate buffer pH 7.2 was used for extraction of enzyme. However, when ferrous sulfate and ascorbic acid were added to enzyme preparation in order to maintain the level of cofactor and protecter, ferrous phosphate precipitated and pH value was lowered consequently. Hence Tris buffer (1/20 M trishydroxymethylaminomethane-HCl buffer pH 7.8) was newly prepared.

Further purification procedure.

As reported in the previous report, calcium-phosphate gel treated enzyme preparation, Fraction V still possessed oxidative activity on protocatechuic acid (PCA). Moreover, it was found that the cell-free extracts of non-induced cells, which were obtained from culture using glucose as a sole carbon source, were also exhibited oxidative activity on protocatechuic acid[4]. So the authors concluded that this fraction contained at least two kinds of enzyme: protocatechuic acid oxidase and gentisic acid oxidase, and the former was constitutive enzyme in *Pseudomonas ovalis* S-5, and the latter was induced by the one of th inducer i.e, *m*–hydroxybenzoic acid (*m*–HBA). It was, therefore, desirable to separate the protocatechuic acid oxidase from gentisic acid oxidase as the first step in the further purification. The crude extracts were filtrated with 0.5 per cent hyflosupercel, and 0.2 volume of 10 per cent streptomycin sulfate was added with vigorous agitation to remove as much nucleic acid in crude extracts as possible. The precipitate resulted by low-speed centrifugation were discarded. The supernatant liquid was fractionated with neutral saturated ammonium sulfate and precipitate at 0.5 saturation was collected, and redissolved in 1/15 M pH 7.4 phosphate buffer with 10^{-3} M ferrous sulfate and ascorbic acid which were added as protectors against enzyme inactivation, and were dialyzed for 2 hours against the same buffer.

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It was then adjusted to pH 7.4 sharply and protamine sulfate (20 mg per ml pH 7.0) was added slowly. The precipitate was removed periodically by centrifugation, and ratio of optical densities ξ 280 mμ / ξ 260 mμ was determined on a suitable dilution of the supernatant with Beckman spectrophotometer. The addition of protamine continued until this ratio reached at least 0.78, although crude extracts had a ξ 280 mμ / ξ 260 mμ ratio of about 0.65. After the protamine treatment the supernatant was treated with calcium phosphate gel. One-tenth volume of the gel was sufficient for a solution of the enzyme containing 7.5 mg of protein per mililiter. The mixture was immediately centrifuged and the supernatant liquid was collected.

As up to this step of purification on procedures, protocatechuic acid oxidase was not separated from the preparation, in order to separate protocatechuic acid oxidase from gentisic acid oxidase, it was then refractionated by ammonium sulfate. The enzyme protein which precipitates between 0.15 and 0.33 saturation was collected, and redissolved in dist. water. This was then adjusted to pH 7.4 with aqueous ammonia, and this preparation was dialyzed for 1 hour against dist. water at pH 7.0, containing 10^{-3} M Fe^{++} and ascorbic acid. It was then retreated with 1.25 volume of calcium phosphate gel, and the mixture was adjusted sharply to pH 7.4 by addition of 1 N acetic acid. After fifteen minutes standing the mixture was centrifuged, then the collected gel was carefully washed twice, with dist. water and with M/150 phosphate buffer, and eluted with one-tenth of the initial volume of pH 7.6 M/15 phosphate buffer solution. The elution was repeated again, and clear transparent straw colored enzyme solution was obtained. The activity of this enzyme solution was calculated to have the Q_{O_2} value (protein) of 8000. Table I shows the specific activity and the yield of the enzymes. As seen in this Table, the yield of the enzyme was about 10 per cent of the original broth in spite of careful treatment. The amount of purified material available was insufficient for further purification. These loss of yield could not be recovered by addition of SH-protector as glutathione, cysteine and ascorbic acid to Fraction VII.

Separation of the two enzymes.

The enzyme fraction between 0.15 and 0.33 saturation of ammonium sulfate oxidized GA and the supernatant liquid oxidized PCA respectively. In our experiment, separated protocatechuic acid oxidase also required ferrous ion as cofactor.

Protocatechuic acid oxidase was already studied by Stanier et al.5) in detail. They have demonstrated that ferrous ions were not required by protocatechuic acid

TABLE I. PURIFICATION OF GENTISIC ACID OXIDASE

Fraction		Volume	Protein		Specific activity	Yield per cent
			mg/ml	total	Q_{O_2} (protein)	
Crude extracts	(I)	200 ml	13.6 mg	2,720 mg	413	100
Treated with Hyflosupercel	(II)	176	12.7	2,366	439	92.5
Treated with Streptomycin	(III)	240	8.8	1,848		
ppt. by $(NH_4)_2SO_4$ 0.5 satu.	(IV)	40	11.4	456	952	38.7
After Ca-Ph. gel negative absorp.	(V)	40	6.0	272	1,412	34.2
After refractionation by $(NH_4)_2SO_4$ 0.15～0.33 satu.	(VI)	20	3.7	72	3,789	24.4
After Ca-Ph. gel positive absorp.	(VII)	9	1.7	15.5	8,002	10.9

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5) R. Y. Stanier and J. L. Ingraham, *J. Biol. Chem.*, **210**, 799 (1954).

oxidase preparation which was extracted from *Pseudomonas fluorescens* A–3–12. This difference may be caused due to the difficulty of the preparation of iron-free enzyme, and does not preclude the possibility that iron is a component of both preparation. In the case of *Ps. fluorescens*, ferrous ions might be released from enzyme protein by the more strict treatment.

Specificity

The oxidation of several aromatic compounds by this enzyme fraction were tested. As seen in Table II the only compound proved to be attacked was GA. Fumarylpyruvic acid which was the intermediate after cleavage of GA[2)] was also tested but showed negative result.

TABLE II. SUBSTRATE SPECIFICITY OF GENTISIC ACID OXIDASE

Substrates	Oxygen uptake
Gentisic acid	67.0 μl
Homogentisic acid	0.0
Protocatechuic acid	0.0
2, 3-Dihydroxy benzoic acid	0.0
α-Resorcilic acid	0.0
β-Resorcilic acid	0.0
o-Hydroxy benzoic acid	0.0
m-Hydroxy benzoic acid	0.0
p-Hydroxy benzoic acid	0.0
Phenylacetic acid	0.0
Catechol	0.0
Fumaric acid	0.0
Pyruvic acid	0.0

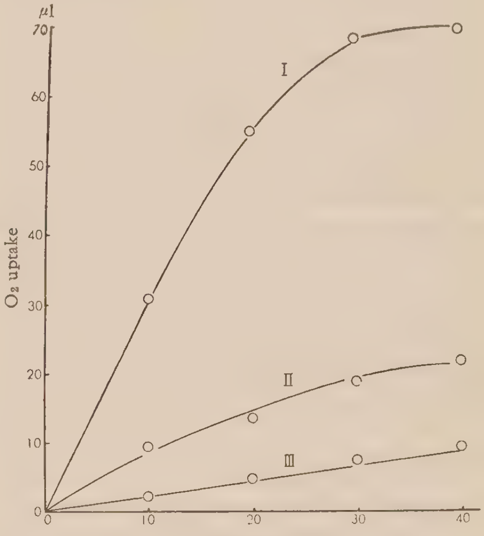
Each substrates were added as sodium salt.
Concentration of each substrates was 3 μM, and activity was indicated by the oxygen uptake for 5 min.

The preservation of enzyme with ferrous ions.

The ferrous ion free enzyme solution was adjusted at pH 7.2 with 1 N HCl and incubated in a water bath regulated at 37°C. On the other hand, 10^{-3} M ferrous ions was added to this preparation and incubated in the same condition. Both samples, incubated for 0, 10, 20, 30, 45, and 60 minutes, were cooled immediately to room temperature and readjusted to pH 8.0 with 1 N NH_4OH. Then activities of these enzyme preparations were determined by use of Warburgs manometer. In the determination, only ascorbic acid was added in the ferrous-enzyme preparation, whereas to the iron-free preparation both ascorbic acid and ferrous ions were added. As seen in Table III, activity of ferrous ion-enzyme preparation didn't remarkably decreased, but loss of the activity of iron-free enzyme occurred with the elapse of time, and anaerobic condition could not prevent the loss of activity.

The combination of iron and enzyme.

Fig. 1 shows the effect of a several combinations of



Time in min.

FIG. 1. Activity of Iron Free Enzyme Preparation preincubated with Ferrous Ions

Curve 1; Preincubated ion-free enzyme with ferrous ion, II; Preincubated iron-free enzyme with substrate, III; Preincubated ferrous ion with substrate.
The reaction was initiated by mixing three components after the preincubation.
Ferrous ion; final 10^{-3} M with ascorbic acid.

TABLE III. THE EFFECTS OF INCUBATION TIME WITH FERROUS ION AT 37°C, pH 7.2

	time	0	10	20	30	45	60	min
I.	Fe⁺⁺ added	100	100	100	98	95	92	activity per cent
II.	not-added	100	82	70	61	52	38	

I; Ferrous Ion 10^{-3}M was added.
Enzyme activity was determined by manometry after incubation.
And in the determination, ascorbic acid 2×10^{-3}M was added to I, and ascorbic acid 2×10^{-3}M with ferrous ion 10^{-3}M to II.

iron free enzyme preparation and ferrous ions. Iron free enzyme was prepared by dialysis of purified enzyme. Three systems were tested; 1) preincubation of the mixture of ferrous ions, ascorbic acid and the enzyme, 11) enzyme and substrate, and 111) ferrous ions, ascorbic acid and substrate respectively. The preincubation was carried out in Warburgs vessels before enzymation. As shown in Fig. 1, when the substrate was added to a preincubated system of ferrous ions, ascorbic acid and enzyme 1), the activities were greater than those of 11) or 111). It was suggested that the formation of a metal-protein complex occurred in the case of 1), and this complex was necessary for the activity of this enzyme. The effects of preincubation were not reported on other benzene ring splitting enzymes.

In the case of aconitase, which required ferrous ion as cofactor as like benzene ring splitting enzymes, it was founded that the ferrous ion-enzyme complex acted as substrates, and this enzyme showed greater activity in the presence of this chelate than the substrate alone.

Action of inhibitors.

Table IV shows the effect of various inhibitors on the enzyme. This enzyme was extremely inhibited by several metal chelators such as α, α'-bipyridyl, *o*-phenanthroline, hydroxyquinoline and nitroso-R-salt, and also by metal ions and KCN. Inhibition per cent of KCN markedly differed depending on the concentration of CN^-, which might be due to the production of cianhydrine.

The inhibition of chelators was recovered by the addition of ferous ion with ascorbic acid 10^{-3} M, but not by the addition of ascorbic acid or other reducing agents alone. Table V shows the effects of chelating reagents and recovery of activity by ferrous ion.

TABLE IV. THE EFFECT OF SEVERAL INHIBITORS

Inhibitor	concentration	inhibition per cent
Monoiodoacetate	1×10^{-3} M	11.2
p-Chloromercury-benzoate	1×10^{-3}	12.6
NaF	1×10^{-2}	4.0
Semicarbazide	1×10^{-2}	17.6
$AgNO_3$	1×10^{-3} 1×10^{-4}	100.0 53.9
$CuSO_4$	1×10^{-3} 5×10^{-4}	60.5 57.3
α, α'-Bipyridyl	1×10^{-3}	80.0
Ethylenediamine-tetraacetic acid	1×10^{-3}	74.0
8-Hydroxyquinoline	1×10^{-3}	100.0
o-Phenanthroline	1×10^{-3}	100.0
KCN	1×10^{-3} 1×10^{-4}	100.0 39.5
NaN_3	1×10^{-3}	29.5

TABLE V. THE EFFECT OF CHELATING REAGENTS AND RECOVERY OF ACTIVITY BY FERROUS ION

Chelating reagent	Inhibition per cent	$Fe^{++}(10^{-3}M)$added recovery per cent
none		100
α,α'-Bipyridyl	50.0	98.0
o-Phenanthroline	38.0	80.0
8-Hydroxyquinoline	46.0	93.0
Nitroso R salt	21.0	75.0

TABLE VI. INHIBITION BY *p*-CHLOROMERCURY-BENZOATE

PCMB Concentration	I E+S+(PCMB)	II (E+PCMB)+S	III (E+S)+PCMB
10^{-4} M	12.0	40.8	7.0
10^{-3} M	46.1	82.4	21.5

Values are shown by inhibition per cent.
E ; enzyme, S ; substrate, PCMB ; *p*-chloromercury-benzoate.
I ; PCMB was adeed at the start of reaction.
II ; PCMB was preincubated with enzyme for 30 min. before reaction.
III ; PCMB was added 7 min. after the start of reaction.

The effect of SH-reagent.

p-Chloromercury benzoate and monoiodoacetic acid in the concentration at 10^{-3} M showed almost no inhibition when they were added at the start of the enzyme reaction. When preincubated with enzyme, however, these substances inhibited enzyme reaction very clearly. These are specific SH-inhibitors, so the activity of this enzyme might be attributed to SH-radical. Table VI shows the activity of enzyme (E) preincubated with *p*-chloromercury-benzoate (PCMB) and with substrate (S) in various combinations. In each case reactions were carried out in the Warburgs vessel at 30° C. The inhibition in the combination (II) was higher than that in the case of (I), irrespective of the PCMB concentration. And in the case of (III), it was always lower than that of (I). It was suggested that the formation of enzyme-substrate complex was related to SH-radical in some manner. This inhibition was recovered to some degree by addition of glutathione, although only a few recovery was found in the case of the enzyme combined with PCMB for a long time.

Furthermore, it was proved that such purified preparation did not contain a spectrophotometrically detectable amount of flavin.

Analogue inhibitions.

Inhibition by the analogues of GA were examined.

TABLE VII. INHIBITION BY ANALOGUE COMPOUNDS OF GENTISIC ACID

Compounds in vessels	concentration	activity O_2 uptake in 30min.
Gentisic acid (G)	2.9×10^{-3}M	61.04
(G) and	2.9×10^{-3}	7.27
Homogentisic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	18.09
Homogentisic acid	2.0×10^{-3}	
(G) and	2.9×10^{-3}	59.8
p-Hydroxybenzoic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	61.0
m-Hydroxybenzoic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	62.5
o-Hydroxybenzoic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	61.0
2, 3-Dihydroxybenzoic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	60.04
Protocatechuic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	64.02
α-Resorcilic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	62.02
β-Resorcilic acid	2.9×10^{-3}	
(G) and	2.9×10^{-3}	61.04
Catechol	2.9×10^{-3}	
(G) and	2.9×10^{-3}	45.9
Resorcin	2.9×10^{-3}	
(G) and	2.9×10^{-3}	9.1
Hydroquinone	2.9×10^{-3}	

As seen in Table VII, none of these compounds including protocatechuic acid were oxidized, which indicates that the Fraction VI was highly specific for the oxidation of GA. The relative afinities of each of these analogues to the enzyme were tested by determining their individual effects on the rate of oxidation to an equimolar concentration of GA. Except hydroquinone (HQ) and homogentisic acid (HGA), which were inhibitory, none inhibited the initial rate of oxygen uptake significantly.

It was very interesting that only HQ and HGA, in which hydroxy radicals occupies the positions of 2 and 5 as GA, inhibited gentisic acid oxidase activity. The inhibitory actions of these compounds, therefore, may be attributable to the similar specific composition between GA and these compounds. The typical competition curve, however, has not been obtained in the present experiment.

DISCUSSION

We assumed that this enzyme was related to other oxidases which act upon phenolic compounds: homogentisic acid oxidase[6~10], pyrocatechase[11~13], protocatechuic acid oxidase[5], since in each case, molecular oxygen is an obligatory oxidant, and two atoms of oxygen are consumed in the reaction, the products are unsaturated di- or tri-carboxylic acid. These four enzymes bring about extremely specific cleavages of the benzene ring. Their known properties are summarized in Table VIII. R. Y. Stanier et al.[5] have reported that neither glutathione nor ferrous ions requirement could be shown at any stage in the purification of protocatechuic acid oxidase. More recently, however, S. Dagley[14] and E. L. Tatum[15] have reported that ferrous ion is required as cofactor for protocatechuic acid oxidase. It was assumed that this striking difference may be quantitaive and does not preclude the possibility that iron in a component of all these enzymes.

The optimum concentration of required ferrous ions for the purified enzyme preparation existed between 10^{-3} M and 10^{-4} M, when examined by Warburgs manometer. However, it is doubtful that whether iron is competely free from the enzyme preparation or not, so that the enzyme should be further purified, if ferrous ions concentration should be exactly determined.

Maleyl-pyruvate, an initial intermediate of the ring fission was recognized as *cis*-form by spectrophotometrical assay of the reaction mixture after 15 minutes incubation using this enzyme. It is believed that GA is oxidized with benzene ring fission to *cis*-form aliphatic compound, maleylpyruvate, and then is transformed to the isomer of *trans*-form, fumarylpyruvate. W. E. Knox et al.[10] have reported that HGA was oxidized to fumarylacetoacetate by glutathione requiring isomerase obtained in cell-free extracts. Our spectrophotometrical experiments

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TABLE VIII. COMPARISON OF THE RING SPLITTING ENZYMES

Enzyme	Inhibition	Reversal of inhibition	Analogue inhibition	Opt. pH	Reference
Gentisic acid oxidase	Dialysis	Fe^{++}+ascorbate	Homogentisic acid	8.0	This paper
	α, α′-Bipyridyl	〃 〃			
	Oxyquinoline	〃 〃			
	o-Phenanthroline	〃 〃	Hydroquinone		1.
	Nitroso R salt	〃 〃			
	PCMB	Fe^{++}+GSH			
	KCN				
Homogentisic acid oxidase	Dialysis	Fe^{++}+ascorbate	Hydroquinone	7.0~7.2	6, 7, 8, 9, 10.
	H_2S, Cystein				
	KCN				
	α, α′-Bipyridyl	Fe^{++}+ascorbate			
	Versene	Fe^{++}			
	Oxine				
	o-Phenanthroline				
	PCMB	Fe^{++}+GSH			
Pyrocatechase	Dialysis	Fe^{++}		8.0	11, 12, 13.
	α, α′-Bipyridyl				
	o-Phenathroline				
	PCMB	GSH			
Protocatechuic acid oxidase	PCMB	GSH	Catechol	probably 9.0	5, 14, 15.
	Nitroso R salt	Fe^{++}+ascorbate	2, 3- and 2, 4-Dihydroxy-benzoic acid		4.
3-Hydroxy-anthranilic acid oxidase	α, α′-Bipyridyl				16.
	Oxine				
	Versene				
	Citric acid				

indicated that *cis*-form intermediate was isomerized spontaneously to the *trans*-form. However, when dialyzed crude enzyme reacted on GA, it only produced the *trans*-form intermediate, so that *cis-trans* isomerization must have been carried out by an isomerase existed in the crude extracts.

SUMMARY

1. A highly purified gentisic acid oxidase preparation free from protocatechuic acid oxidase was obtained by the repeated ammonium sulfate precipitations. The specific activity increased up to twenty-fold. This enzyme preparation is free from oxidases of other aromatic compounds, as maleylpyruvate isomerase and fumarylpyruvate hydrolyase.

2. The activity of this enzyme preparation was remarkably inhibited by metal reagents such as α, α′-bipyridyl, ethylenediaminetetraacetic acid, o-phenanthroline and nitroso-R-salt, and the inhibition was reversed by the addition of ferrous ions, and reactivation increased by the further addition of reducing agents, but not by the reducing agent such as ascorbic acid alone.

3. Enzyme and ferrous ion combination was proved to be necessary for the activity of the gentisic acid oxidase as the results of experiments using several combinations of iron-free enzyme, substrate and ferrous ions.

4. The loss of activity of iron-free enzyme could be recovered by the addition of ferrous ion. Other metalic ions and reducing agents tested were not effective.

5. Participation of SH-radical in the enzyme-substrate combination was presumed from the inhibition pattern by SH-reagents such as *p*-chloromercury-benzoate.

The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continued guidance throughout this research.

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16) M. H. Mehler, *J. Biol. Chem.*, **218**, 241 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 255~261, 1960]

Metabolism of Aromatic Compounds by Microbes

Part IX. The Enzymatic Conversion of Gentisic Acid to Fumarylpyruvic Acid

By Shin-ich SUGIYAMA, Hirosato TANAKA, Keiji YANO and Kei ARIMA

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

Received August 17, 1959

One of the presumed product after the ring cleavage of gentisic acid by the gentisic acid oxidase free of hydrolysing enzyme from *Pseudomonas ovalis* S-5 was obtained in a crystalline form. This new substance was proved to be a β-diketodicarboxylic acid and was identified as fumarylpyruvic acid, as a results of investigation of the chemical and physical properties. Furthermore, maleylpyruvic acid, the initial product of the ring cleavage of gentisic acid oxidation was recognized in the reaction mixture using the purified enzyme. This is proved to be transformed to fumarylpyruvic acid, which then was hydrolyzed into pyruvic acid and fumaric acid by crude cell free extract. Thus the pathway of gentisic acid oxidative metabolism was established.

The authors had already investigated the metabolism of *m*-hydroxybenzoic acid by *Pseudomonas ovalis* S-5[1]. This acid was oxidized to gentisic acid and then the oxidative ring cleavage of gentisic acid was ocurred[2]. The enzyme which catalized the reaction of the gentisic acid oxidation named gentisic acid oxidase was obtained[2] as the cell-free preparation and its purification, its properties were studied and described in the previous reports[3] in which the authors presumed from several experimental results that ring cleavage of gentisic acid may occur between carbons 1 and 2 giving rise a β-diketo-dicarboxylic acid: fumarylpyruvic acid or maleylpyruvic acid (Fig. 1).

Recently we have succeeded in isolating one of the direct ring cleavage product in a crystalline form. From the results obtained the crystalline intermediate was identified as fumarylpyruvic acid. The presence of a *cis* intermediate maleylpyruvic acid was also confirmed spectrophotometrically in the reaction mixture using purified enzyme preparation.

EXPERIMENTS AND RESULTS

Enzyme preparation *Pseudomonas ovalis* S-5 was grown in bouillon peptone medium for 18 hrs. with constant shaking, harvested after this by centrifugation and washed with dist. water. The resting cells thus obtained were induced by *m*-hydroxybenzoic acid for three hrs. in Sakaguchi flasks at 30°C[4].

The induced cells harvested by centrifugation were ground by hand with the same weight of alumina until breakage occured.

The mixture was suspended in 2 ml of 0.05 M Tris-hydroxyaminomethane-HCl buffer (pH 7.8) per gm. of wet cells and the bulk of the alumina was removed by low speed centrifugation. The extract was centrifuged at 10,000×g for 30 minutes to remove unbroken cells. All procedure was carried out in the cold room (2°C±2°). For the purpose of obtaining the ring cleavage product it was only necessary to remove the hydrolysing enzyme. The accumulation of the intermediate in the reaction mixtures using non-dialyzed extracts for 20 hrs., 40 hrs., and more than 40 hrs. were carried out, and the dialyzed crude extracts were investigated with *o*-phenylenediamine reagent. *o*-Phenylene-

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[1] S. Sugiyama, K. Yano, K. Komagata, M. Kazama and K. Arima, This Bulletin, **24**, 211 (1960).

[2] S. Sugiyama, K. Yano, K. Komagata and K. Arima, This Bulletin, **24**, 243 (1960).

[3] S. Sugiyama, K. Yano and K. Arima, This Bulletin, **24**, 249 (1960).

[4] K. Arima, K. Komagata, S. Sugiyama, K. Kazama and K. Yano, This Bulletin, **19**, 51 (1954).

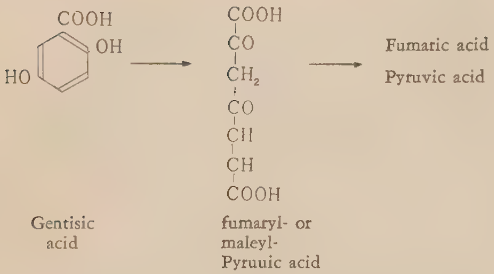

FIG. 1. Pathway of Oxidative Metabolism of Gentisic Acid

diamine reagent were prepared according to the methed described by R. F. Witter et al.[5] The reactions were carried out for 30 min. in Warburg's vessel containing 0.5 ml enzyme preparation, 0.5 ml M/10 tris buffer pH 7.8, 0.3 ml M/10 sodium gentisate, and 0.1 ml M/100 ferrous sulfate and ascorbic acid mixture. Half volume of trichloroacetic acid was added to the reaction mixture and then centrifuged at 3,000 r. p. m. for 15 minutes. 0.25 ml of the supernatant was diluted to 6 ml with dist. water and 1 ml of *o*-phenylenediamine reagents was added. Then these were allowed to stand for 30 min. at room temperature. The optical densities of reaction mixtures with *o*-phenylenediamine were measured by HITACHI spectrophotometer model 40 P.

The more the crude enzyme extracts dialyzed, the more the optical density of reaction mixture with *o*-phenylenediamine increased. However, no inrease was observed after 40 hrs. dialysis. So the enzyme preparation for the accumulation of the intermediate could be obtained by dialyzing the crude extracts for 50 hrs against dist. water which was adjusted to pH 8.0 with aqueous ammonia.

Isolation and purification of the intermediate.

The dialyzed crude enzyme extracts prepared as described above was diluted 8 folds with M/10 Tris-aminomethane-HCl buffer pH 8.0

The enzymatic oxidation was carried out in 500 ml Sakaguchi flask containing 50 ml of M/10 sodium gentisate, 5 ml of 10^{-2} M aqueous solution of ferrous sulfate and ascorbic acid, and a small quantity of sodium bicarbonate, with constant shaking for 30 to 40 minutes. The pH of the reaction mixture without sodium bicarbonate would go down as low as 5 during the incubation and the oxidation reaction would stop accordingly, leaving not a small quantity of sodium gentisate unoxidized. After 40 minutes incubation, no sodium gentisate was detected in the reaction mixture by Folin's phenol reagent.

The reaction mixture was deproteinized with 0.1 volume of 20 per cent metaphosphoric acid and centrifuged. The supernatant was extracted continuously with peroxide free ethyl-ether for about 50 hrs. The ether was removed from the extract under reduced pressure in a desiccator. Then yellow crude intermediate was obtained. The yellow crude intermediate was dissolved in ethylacetate and then petrolium ether was added until slight precipitation was found. This was allowed to stand at −20°C over a night. Slightly yellow precipitate thus obtained was made free of ethylacetate and petroleum ether under reduced pressure and then dissolved in a small amount of ethanol. More than 10 times benzene to ethanol in volume was added to the almost saturated ethanol solution. Then it was stood under reduced pressure with special precaution. It was discontinued just before the solution became turbid. The crystallization did not occur by merely adding benzene dropwise to the saturated ethanol solution. The crystalline intermediate appeared after standing it at 4∼5°C over a night of two. It was recrystallized three times from ethanol-benzene mixture as described above.

Properties of the intermediate.

Elementary analysis An analytical sample was prepared by careful drying of the pure intermediate with an Abderhalden aparatus.

Anal. Calcd. for $C_7H_6O_6$; C 45.17 ; H 3.25
Found C 45.20 ; H 3.40
dec. P. 180∼181°C

This intermediate geve a sharp yellow color reaction with *o*-phenylenediamine. It also readily reacted with carbonyl reagents such as sodium nitroprusside, 2, 4-dinitrophenylhydrazine and semicarbazide.

Titration of the intermediate. Direct evidence for the dicarboxylic and enolic nature was obtained by titrating with alkali with use of glass electrode. 45 mg of the intermediate was dissolved in 3 ml of ethanol and 1 ml of water and titrated with M/10 sodiumhydroxide. The titration curve (Fig. 2) showed that there were two carboxyl groups ; their pK values appear in this condition to be at 3 and 4.4 respectively.

Further titration up to pH 12 revealed the presence of an additional ionizable group with pK at 7.8 (presumably representing the enolic form of the β-diketo group).

UV spectra of the crystalline intermediate. As shown in Fig. 3, this compound had a strong absorption

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5) R. F. Witter and E. Stotz, *J. Biol. Chem.*, **176**, 501 (1948).

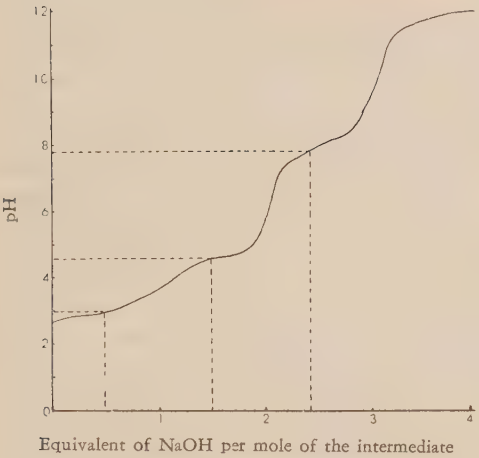


FIG. 2. Titration Curve of the Intermediate

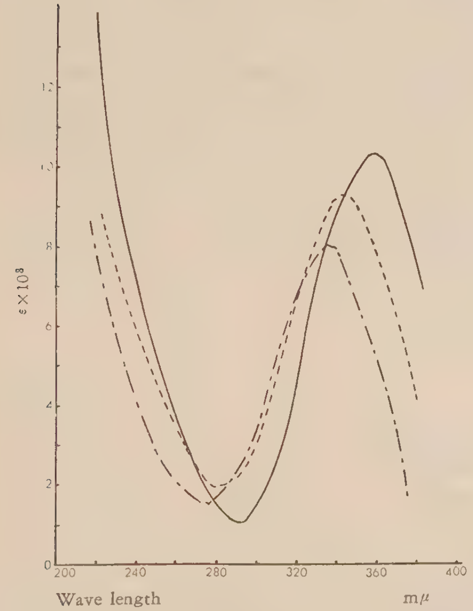


FIG. 3. Ultraviolet Absorption Spectra of the Intermediate

——— pH 13
– – – – – pH 7.5
— - — - — pH 1.0

near 340 mμ which slightly shifted when pH was raised. The maximum molar extinction coefficients for this compound were 8,300 at 335 mμ in 0.1 N HCl; 9,400 at 340 mμ in phosphate buffer at pH 7.3; 10,300 at 350 mμ in 0.1 N NaOH. These facts were also compatible with the presumed structure of fumarylpyruvic acid. Judging from these molar extinction coefficient values, we should presume that most of this acid exists in *mono*-enol form even in acidic side.

Infra-red spectrum of the intermediate. Infra-red spectrum of this compound in Nujol mull is shown in Fig. 4. A deep absorption band at 1,710 cm^{-1} was considered to be due to C=O of unsaturated carboxyl radical. A characteristic broad band in the range 1,630～1,500 cm^{-1} was attributed to the conjugated chelation of *mono*-enol β-diketone structure.

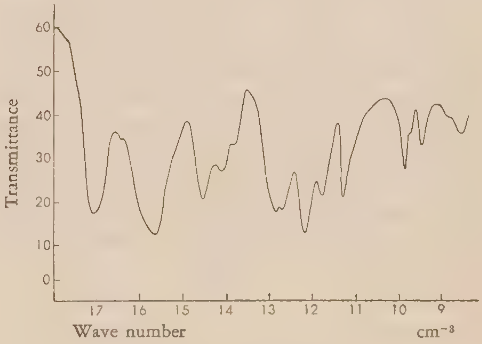


FIG. 4. The Infra-red Spectrum of the Intermediate

The sharp band at 980 cm^{-1} was assigned to the out-of-plain deformation vibration of the hydrogen atoms of *trans*-ethylenic linkage.

Enzymatic hydrolysis of the intermediate. A further fact to confirm the structure of fumarylpyruvic acid and its metabolic fate in this *Pseudomonas* strain was obtained by hydrolyzing the intermediate by the crude enzyme extracts prepared from the *m*-HBA induced cells.

As described above, the crude extract of *m*-HBA induced cells which had not yet dialyzed contained the fumarylpyruvic acid hydrolyase which hydrolyzes fumarylpyruvic acid.

The crude extracts were free of the particulate fraction by centrifugation at 20,000×g. So the crude extract was thought to be free of the cyclophorase. This fact was also confirmed by estimating the oxidizability of this extract toward fumaric acid, succinic acid, and maleic acid. None of these compounds were oxidized.

5 mg of pure intermediate was dissolved in M/10 Tris-aminomethane-HCl buffer at pH 7.8 and added to 5 ml of non-dialyzed crude extracts to final volume of 10 ml, and was incubated at 30°C for 1.5 hrs. After the incubation, no *o*-phenylenediamine positive substance was

detected in the reaction mixture. The reaction mixture was deproteinized with metaphosphoric acid and then adjusted at pH 2.0 with 1 N H_2SO_4 and continuously extracted with peroxide free ether.

The acids in the extract were identified chromatographically on the paper sprayed with 0.2 per cent bromophenol blue, using the upper layer of butanol 4 : acetic acid 1 : water 3, as its developing solvent. The extracts yielded two distinct acidic spots which showed the same R_F values with fumaric acid and pyruvic acid respectively.

The same procedures were carried out with 30 μM of GA as the substrate, and without substrate. When gentisic acid was used as the substrate, the result obtained by paperchromatography was the same as in the case of the fumarylpyruvic acid was used as the substrate. No spot was detected by spraying B. P. B. (bromopheenol blue) solution except two, fumaric acid and pyruvic acid (Fig. 5). When the crude extracts were incubated without substrate, no acidic spot was detected at all. These facts showed that GA was metabolized via the intermediate into fumaric and pyruvic acids, and the crystalline intermediate is 7 carbon fumarylpyruvic acid. Physical and chemical properties of this new substance, fumarylpyruvic acid were summerized in Table I.

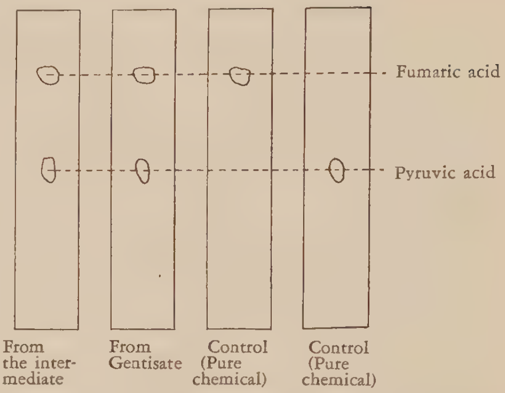
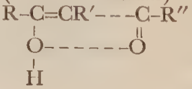


FIG. 5. Paperchromatogram of the Enzymatic Hydrolysates of the Intermediate

Detection of *cis* form intermediate, meleylpyruvic acid.

To know the existence of the *cis* form intermediate, the authors employed the purified preparation of gentisic acid oxidase, described in the previous report, which was free of the isomerase converting *cis* intermesiate into *trans*

TABLE I. PROPERTIES OF THE NEW SUBSTANCE, FUMARYLPYRUVIC ACID

Formula	$C_7H_6O_6$ *anal.* calcd. C: 45.17, H: 3.25, O: 51.28 found. C: 45.20, H: 3.40	
Molecular weight	186.12	
Crystalline form	Bipyramid (in ethanol-benzene)	
Color	Slightly yellow	
m.p. (dec.)	180∼181°C	
pK	$pK_1=3.0$, $pK_2=4.4$, $pK_3=7.8$ (in ethanol 3 : water 1)	
Positive reagents	2, 4-Dinitrophenylhydrazine Semicarbazide Sodium-nitroprusside *o*-Phenylenediamine	
Ultraviolet absorption	pH 1 ; 335 mμ (ε=8,300), pH 7.5 ; 340 mμ (ε=9,400), pH 13 ; 350 mμ (ε=10,300),	
Infrared absorption	C=O Vibration, α-β-unsaturated acid	1,710 cm^{-1}
	C—O– Stretching vibration,	1440∼1935
	C=C Stretching vibration	1320∼1211
	C=C Conjugated	near 1600
	C—H Out of plane deformation vibration (-CH=CH- *trans*)	980
	 $\mathrm{R{-}\underset{\underset{\underset{H}{|}}{O}}{\underset{|}{C}}{=}CR'{-}{-}{-}\underset{\underset{O}{\|}}{C}{-}R''}$ Conjugated chelation, monoenol-diketone	1560∼1570

form fumarylpyruvic acid.

The enzymatic oxidation was carried out in a Warburg's vessel containing 3 μM of GA, 0.25 ml of the purified enzyme solution, 0.75 ml of Tris buffer with 0.1 ml of ferrous sulfate and ascorbic acid solution (10^{-3}M) at 30°C for 15 min. The reaction mixture was deproteinized immediately with 0.5 ml of 10 per cent trichloroacetic acid, centrifuged, and the precipitate was discarded. And the supernatant was neutralized with sodium hydroxide solution. The absorption spectra of this preparation is shown in Fig 6. The reaction mixture absorbed strongly near3 40 mμ at pH 13 and it showed but a very weak absorption at pH 1.5. The absorption in acid solution at 340 mμ increased when it was allowed to stand at pH 1.5 at room temperature (see Fig. 6). These results might be due to the fact that the first degradation product of GA oxidation was maleylpyruvic acid which absorbed strongly only in alkaline solution and then the maleylpyruvic acid was converted into the *trans* intermediate, fumarylpyruvic acid, which absorbed strongly both in alkaline and acid solutions. This conversion was conducted probably by an enzyme in crude extracts, but not by merely standing in an acid solution at room temperature.

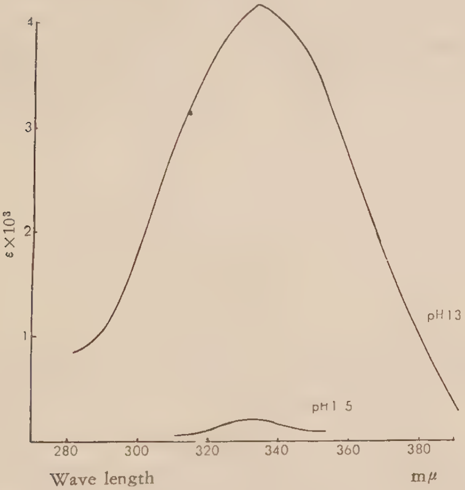


FIG. 6. Ultra Violet Absorption of the *cis* Isomer Maleylpyruvic Acid

Determination of fumarylpyruvic acid.

Fumarylpyruvic acid was determined principally by the method of R. F. Witter et E. Stotz⁵⁾ which was the colorimetric determination of β-diketone using *o*-phenylenediamine. Since values by their method were not always linear function, a modification of it was carried out.

An amount of 100 mg *o*-phenylenediamine is dissolved in 25 ml phosphate-sulfric acid reagent, freshly prepared daily. A sample which contained about 20～100 μg of fumarylpyruvic acid was filled up to 10 ml with dist. water and added 2 ml of *o*-phenylenediameine reagent, allowed to stand at room temperature for 30 min. The aquous solution was transported to 30 ml volume separating funnel and shaked with 10 ml ethylether. The substance which was produced by the reaction between fumarylpyruvic acid and *o*-phenylenediamine moved into upper ether layer, but excess *o*-phenylenediamine remained in aquous layer, which was discarded. 10 ml of 14 per cent Na_2CO_3 aquous solution was added to ether solution and shaked in the separating funnel. Yellow substance, then moved to aquous layer. And this color was measured by spectophotometrically at 440 mμ. Determination was carried out with water as control. Relation between optical density and concentration of fumarylpyruvic acid is shown in Fig. 7. Pyruvic acid gives no infulence to this determination.

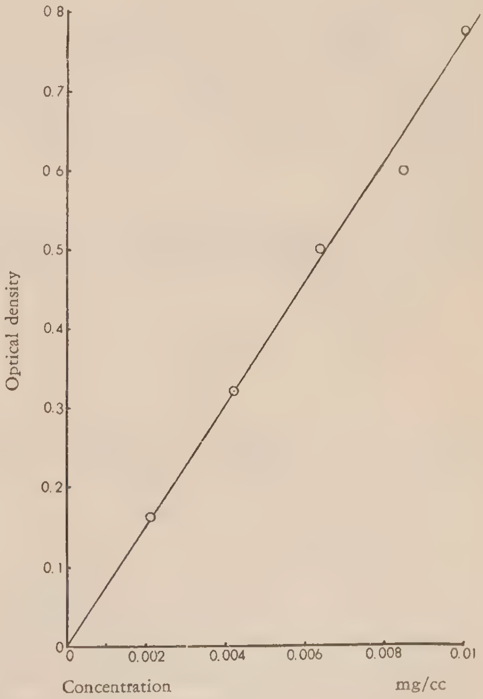


FIG. 7. Relation between optical Density and Concentration of Fumarylpyruvic acid

Condition of determination is discribed in this paper.

DISCUSSION

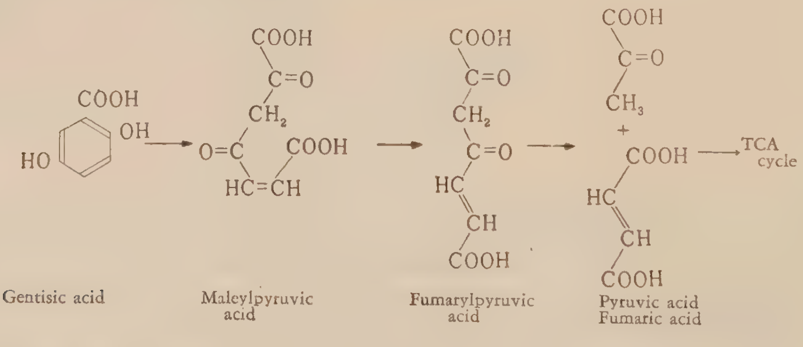
The ultraviolet absorption spectrum of β-diketone shows the maximal spectrum near 330 mμ in alkaline solution. A. Meister reported that characteristic absorptions of triacetic acid and other β-diketones are enhanced by alkali, and suppressed by acid[6,7].

Fumarylpyruvic acid which absorbes strongly both in acid and alkaline solutions has a similar spectra reported for fumarylacetoacetic acid by R. G. Ravdin et al[8], who reported that the striking pH stability of the spectrum appeared to be a reasonable property for fumarylacetoacetic acid on theoretical grounds. The fumaryl double bond of fumarylacetoacetic acid forms as conjugated system with either the keto or enol form of the δ-carbonyl group. Succinylacetoacetic acid which is saturated in the double bond of fumarylacetoacetic acid with hydrogen, shows almost identical spectrum with that of triacetic acid.

Fumarylpyruvic acid, as mentioned above, shows very similar absorption spectra to that of fumarylacetoacetic acid, forms the conjugated double bond system and was presumed that most of this acid exists in *mono*-enol form even in acidic side.

W. E. Knox et al.[9] reported that maleylacetoacetic acid which absorbs strongly in alkaline solution only, is initial product of homogentisic acid oxidation. In the gentisic acid oxidation, *cis*-isomer of fumarylpyruvic acid is expected to exist prior to fumarylpyruvic acid which is the *trans*-form, since the original configuration around these carbon atoms in the benzene nucleus, gentisic acid, is necessarly of *cis* form. The result of spectrophotometrical assay indicates quite enough that maleylpyruvic acid, *cis* isomer of fumarylpyruvic acid, is the direct ring cleavage product. It might be impossible to isolate this acid in a pure state, since this acid converts spontaneously into *trans*-form, particularly, when it is allowed to stand in acidic side. *Cis-trans* isomerization between both isomerization between both iosmeric acids is considered to be catalyzed by enzymes included in crude extracts, since the purified enzyme produces maleylpyruvic acid and the dialyzed crude enzyme accumulates fumarylpyruvic acid, respectively.

The evidence for existence of two intermediates, the one is fumarylpyruvic acid obtained in a crystalline form and identified, and the other is maleylpyruvic acid recognized spectrophotometrically, establishes the oxidative metabolism of gentisic acid by *Pseudomonas ovalis* S-5 as follows.



SUMMARY

1) One of the presumed intermediates of the gentisic acid oxidation was obtained in a crystalline form using dialyzed crude gentisic acid

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[6] A. Meister, *J. Biol. Chem.*, **178**, 577 (1949).

[7] A. Meister and J. P. Greenstein, *J. Biol. Chem.*, **175**, 573 (1948).

[8] R. G. Ravdin and D. I. Crandall, *J. Biol. Chem.*, **189**, 137 (1951).

[9] W. E. Knox and S. W. Edward, *J. Biol. Chem.*, **216**, 489 (1955).

oxidase, free from fumarylpyruvic acid hydrolyase, from the cells of *Pseudomonas ovalis* S-5.

2) From the results obtained, the crystalline intermdediate was identified as fumarylpyruvic acid, which was splitted by crude extracts into pyruvic and fumaric acids.

3) Maleylpyruvic acid, the initial product of gentisic acid oxidation, was recognized spectrophotometrically in the reaction mixture using purified enzyme. And the pathway of gentisic acid oxidative metabolism was established.

4) Colorimentric determination of fumarylpyruvic acid is also described.

The authors wish to express their sincere thanks to Honorary Prof. K. Sakaguchi for his continued guidance throughout this research.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 261~265, 1960]

Studies on the Organophosphorus Insecticides

Part I. Synthesis and Toxicity of Phosphates Containing Vinyl Radical

By Yoshihiko Nishizawa

Laboratory of Sumitomo Chem. Co., Ltd., Osaka

Received May 19, 1959

Many phosphates containing vinyl radical such as Phosdrin[1] were prepared in order to study the correlation between structure and toxicity. From the results obtained it seems certain that in regard of toxicity, it is the carboalcoxy radical rather than the vinyl radical that is necessary and furthermore the toxicity is connected with the atom or the radical linked with the vinyl radical. As a results of these studies, some new toxic phosphates were synthesized.

As it is well known, all organophosphorus compounds do not always show toxicity and insecticidal activity but are highly toxic when they have a definite structure.

For instance take Phosdrin[1], it is found that the introduction of the carboalcoxy-vinyl radical to the phosphate gives toxic property, though trialkyl phosphates have no toxicity.

Therefore, the studies on the correlation between toxicity and the structure is of a fundamental importance in the application of phosphates for agricultural use.

From studies on the chemical constitution of phosphates containing vinyl radical such as phosdrin, it becomes clear that the thiono-form is not toxic[2] and the *cis*-configuration has a higher toxicity than that of the *trans*[3].

So far nothing has been reported on the influences of the substituents in the Phosdrin-type phosphates. Therefore, an attempt to study the chemical structure giving toxic property on

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[1] A. R. Stiles, U.S.P. 2,685,552 (1954).

[2] G. Schrader, BIOS Final Report, No. 714 (1947).

[3] J. E. Casida, *Science*, **122**, 497 (1955).

TABLE I. THE TOXIC DOSE OF PHOSPHATES (LD_{50})

No.	Formula	Oral Toxicity of Mice	
		mg/kg	Mol/kg
I.	$(CH_3O)_2P(O)OC(CH_3):CHCOOC_2H_5$	7.8	3.2×10^{-5}
II.	$(CH_3O)_2P(O)OC(CH_3)(Cl)CH(Cl)COOC_2H_5$	22.0	7.1×10^{-4}
III.	$(CH_3O)_2P(O)OC(CH_3):CClCOOC_2H_5$	60.0	2.2×10^{-4}
IV.	$(CH_3O)_2P(O)OC(CH_3):C(C_6H_5)COOC_2H_5$	32.0	1.0×10^{-4}
V.	$(CH_3O)_2P(O)OC(CH_3):\underset{\overset{\mid}{CH_2COOC_2H_5}}{C}-COOC_2H_5$	125.0	3.9×10^{-4}

Phosdrin was made.

Phosdrin has two characteristic parts, namely, the vinyl and carboalcoxy-radical. Therefore, in these studies, influences of these two radicals on the toxicity were examined one by one.

1) The influence of the vinyl radical

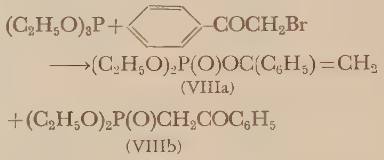
The chlorination of O,O-dimethyl-O-(2-carboethoxy-1-methyl-vinyl)-phosphate (I) proceeded in a smooth way and the dichloro-derivatives (II) prepared were obtained in a good yield. It is clearly shown that two chlorine atoms add to the vinyl radical, because this dichloro-derivative already has no infrared absorption of the vinyl radical (see Fig. 3). The toxicity of this dichlorophosphate (II) is roughly a half-time weaker than the parent phosphate (I) (see Table I). Furthermore, a marked reduction in toxicity occurs when radicals such as halogen (III), phenyl (IV) or carboethoxy-methyl radical (V) are substituted in the α-position of the carboethoxy-radical of Formula (I) as shown in Table I.

The C=C absorption of ethylene in the parent phosphate (I) at 1660 cm^{-1} shows only a small frequency shift to 1650~1630 cm^{-1} with a α-substitution (for example, see Figs. 1 and 2.). Though reasons for this shift are not clarified this shift must indicate an elognation of the double bond under the influence of the α-substituted atom or radicals.

From the above results it is possible to consider that the substitution of the hydrogen atom of vinyl radical or the saturation of the vinyl radical by chlorine, reduces toxicity; in other words, the vinyl radical has some sensitive influence to toxicity.

2) The influence of the carboalcoxy radical.

In order to study the influence of the carboalcoxy-rdical, O,O-diethyl-O-(1-phenyl-vinyl)-phosphate[4] containing no carboalcoxy group was prepared according to the following equation:


$(C_2H_5O)_3P+$
$-COCH_2Br$
$\longrightarrow(C_2H_5O)_2P(O)OC(C_6H_5)=CH_2$
(VIIIa)
$+(C_2H_5O)_2P(O)CH_2COC_6H_5$
(VIIIb)

This process was always acompanied with the keto-phosphate (VIIIb) as the by-product, but it could separate the vinyl-phosphate (VIIIa) from the keto-phosphate (VIIIb) by fractionated distillation just as in the Kreutzkamp meteod[5].

As mentioned above, the vinyl radical has some sensitive influence to the toxicity, but toxicity still remains to some extent by upon saturation. In the case of carboalcoxy-radical, the substitution of carboalcoxy-radical by the hydrogen atom or the phenyl-radical counteracts toxicity (see Table II). Therefore as a consequence of these features, it is obvious that the toxicity depends upon the carboalcoxy radical rather than the vinyl radical.

3) The influence of the substitution of carboalcoxy-radical by some new radicals.

It is very interesting that the substitution of carboalcoxy-radical by chlorine atom shows a slight toxicity (see Table II), though this is not observed in the substitution by hydrogen atom (VIIIa) or phenyl radical (IX).

These results suggest the possibility that new toxic phosphates will be prepared by the sub-

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[4] Arbuzov, *Zhur Abshchim.*, **4**, 834 (1934).
[5] N. Kreutzkamp, *Ber.*, **89**, 1614 (1956).

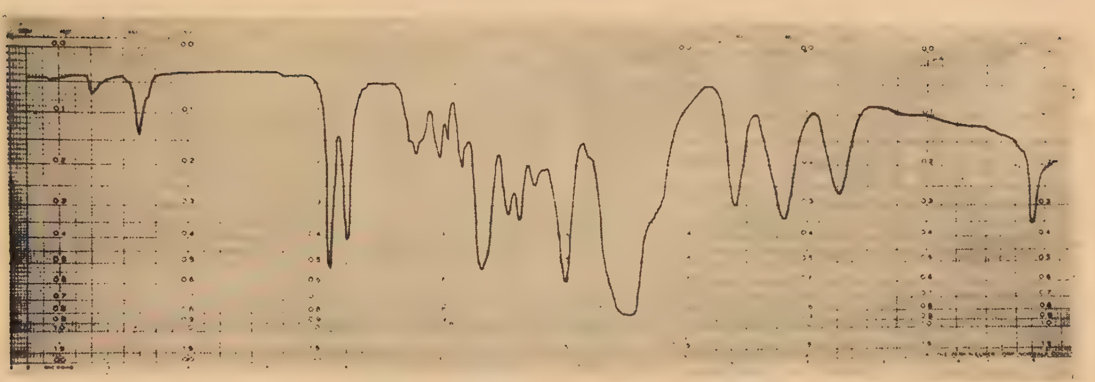


FIG. 1. Infrared Absorption Spectrum of O, O-Dimethyl-O-(2-carboethoxy-1-methyl-vinyl)-phosphate (I)

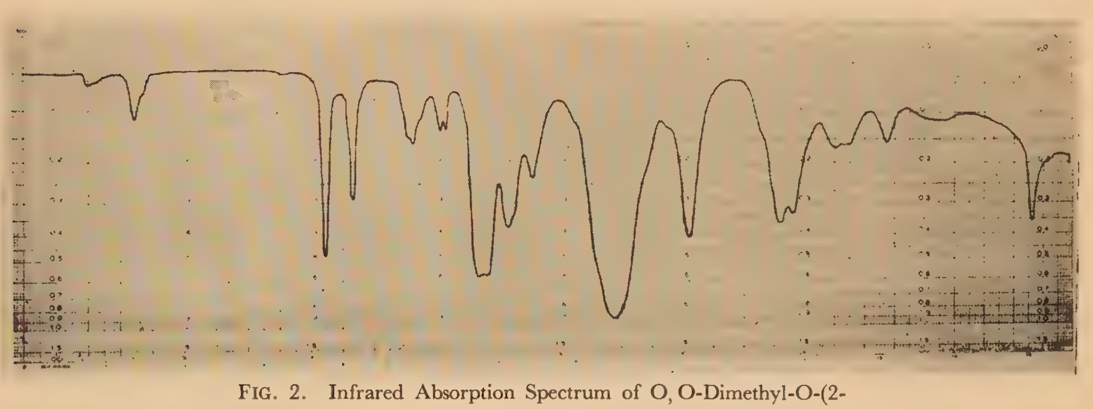


FIG. 2. Infrared Absorption Spectrum of O, O-Dimethyl-O-(2-carboethoxy-2-chloro-1-methyl-vinyl)-phosphate (III)

TABLE II. THE TOXIC DOSE OF PHOSPHATES (LD_{50})

No.	Formula	Oral Toxicity of Mice mg/kg	 Mol/kg
VI.	$(CH_3O)_2P(O)OC(C_6H_5):CHCOOC_2H_5$	11.7	3.9×10^{-5}
VII.	$(C_2H_5O)_2P(O)OC(C_6H_5):CHCOOC_2H_5$	24.0	7.3×10^{-5}
VIIIa.	$(C_2H_5O)_2P(O)OC(C_6H_5):CH_2$	>1000	–
IX.	$(CH_3O)_2P(O)OC(C_6H_5):CH(C_6H_5)$	>1000	–
X.	$(CH_3O)_2P(O)OC(C_6H_5):CHCl$	240.0	9.1×10^{-4}
XI.	$(C_2H_5O)_2P(O)OC(C_6H_5):CHCl$	138.0	4.8×10^{-4}

TABLE III. THE TOXIC DOSE OF PHOSPHATES (LD_{50})

No.	Formula	Oral Toxicity of Mice mg/kg	 Mol/kg
XII.	$(CH_3O)_2P(O)OC(C_6H_5):CH(OC_6H_4NO_2)$	42.0	1.2×10^{-4}
XIII.	$(C_2H_5O)_2P(O)OC(C_6H_5):CH(OC_6H_4NO_2)$	16.6	4.2×10^{-5}
XIV.	$(CH_3O)_2P(O)OCH:CCl(OC_6H_4NO_2)$	20.0	6.2×10^{-5}
XV.	$(CH_3O)_2P(O)OC(CH_3):CHCOCH_3$	55.0	2.6×10^{-4}
XVI.	$(C_2H_5O)_2P(O)OC(CH_3):CHCOCH_3$[6]	24.0	1.0×10^{-4}

TABLE IV. THE YIELD AND THE PHYSICAL AND THE MICROANALYTICAL DATA OF NEW PHOSPHATES

No.	Formula	Yield	B.P.			Phosphorus %		Chlorine %	
		%	°C	mmHg	n_D/t°C	Calcd.	Found.	Calcd.	Found.
I.	$(CH_3O)_2P(O)OC(CH_3):CHCOOC_2H_5$	58	118	1.0	1.4450/28	13.0	12.8	–	–
III.	$(CH_3O)_2P(O)OC(CH_3):CClCOOC_2H_5$	73	138～140	0.8	1.4620/26	11.4	11.8	13.03	13.21
IV.	$(CH_3O)_2P(O)OC(CH_3):C(C_6H_5)COOC_2H_5$	66	147～152	0.35	1.5070/23.5	9.8	10.0	–	–
V.	$(CH_3O)_2P(O)OC(CH_3):\underset{CH_2COOC_2H_5}{\underset{\vert}{C}}COOC_2H_5$	42	160	1.0	1.4549/26.5	9.6	9.3	–	–
VI.	$(CH_3O)_2P(O)OC(C_6H_5):CHCOOC_2H_5$	65	149～151	0.65	1.5259/24	10.3	10.6	–	–
VII.	$(C_2H_5O)_2P(O)OC(C_6H_5):CHCOOC_2H_5$	69	151～153	0.35	1.5140/22.5	9.5	9.7	–	–
IX.	$(CH_3O)_2P(O)OC(C_6H_5):CH(C_6H_5)$	42	165～166	0.15	1.5795/25	10.2	10.4	–	–
X.	$(CH_3O)_2P(O)OC(C_6H_5):CHCl$	51	125～135	0.3	1.5312/28	11.8	11.7	13.52	13.26
XI.	$(C_2H_5O)_2P(O)OC(C_6H_5):CHCl$	61	130～139	0.4	1.5163/24	10.7	11.0	12.22	12.51
XV.	$(CH_3O)_2P(O)OC(CH_3):CHCOCH_3$	55	99～100	0.8	1.4500/25.5	14.9	15.3	–	–
XVI.	$(C_2H_5O)_2P(O)OC(CH_3):CHCOCH_3$	68	101～103	1.0	1.4565/19.5	13.1	13.2	–	–

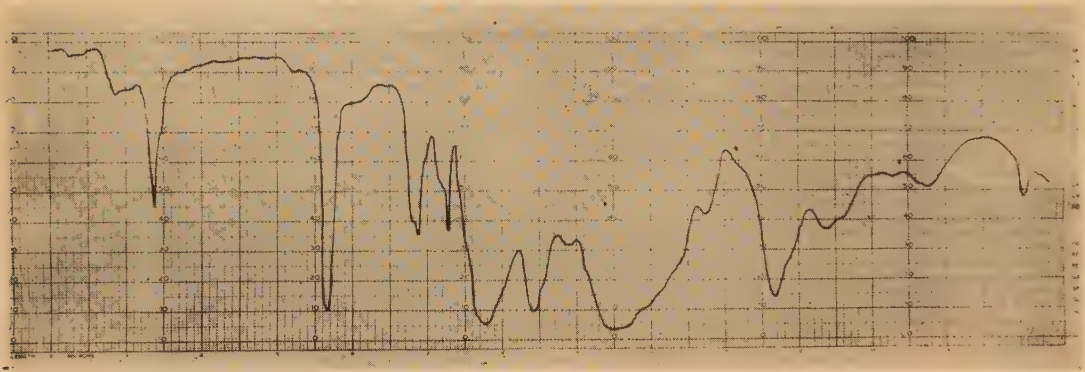
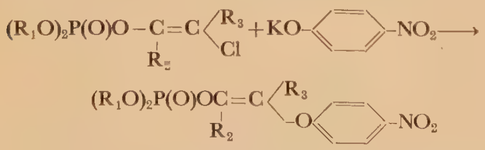


FIG. 3. Infrared Absorption Spectrum of O, O-Dimethyl-O-(1, 2-dichloro-1-methyl-2-carboethoxy-ethyl)-phosphate (II)

stitution of carboalcoxy-radical by some other atom or radical. Thus, an attempt to link the substituents such as *p*-nitrophenoxy- or acetyl-radical with the vinyl radical was made in accordance with the following equations:



$$(R_1O)_3P + CH_3COCHClCOCH_3 \longrightarrow (R_1O)_3P(O)OC(CH_3):\ CHCOCH_3$$

In practice, it was found that these phosphates (XII, XIII, XIV, XV and XVI) have a high toxicity as shown in Table III.

EXPERIMENTAL

1) The preparation of phosphates containing vinyl radical.

The new phosphates were prepared by the reaction of trialkyl-phosphite and α-halo or α, α'-dihalo-keto-compounds in absolute ether or benzene. The yield, physical and the microanalytical data are shown in Table IV, respectively.

2) The chlorination of O,O-dimethyl-O-(1-methyl-2-carboethoxy-vinyl)-phosphate (I)

Sixteen grams (0.05 mol) of O,O-dimethyl-O-(1-methyl-2-carboethoxy-vinyl)-phosphate (I) was added with stirring to 30 ml of chloroform, saturated with chlorine at 10°C, with sufficient cooling to keep the temperature at 0°C.

After standing over night at room temperature, chloroform was evaporated under a reduced pressure and the residual oil was distilled. The fraction boiled at 133～135°C/0.88 mmHg. n_D^{27} 1.4545, Yield 9.0 g (58.1%)

Anal. Calcd. for $C_8H_{15}O_6Cl_2P$; P, 10.1 ; Cl, 22.98%
Found ; P, 9.5 ; Cl, 22.73%.

3) The Synthesis of O,O-dimethyl-O-(1-phenyl-2-*p*-nitro-phenoxy-vinyl)-phosphate (XII).

The phosphate (XII) was prepared by adding 17.7g (0.1M) of *p*-nitrophenol potassium salt into 70 ml chlorobenzene containing 26.0 g (0.1M) of O,O-dimethyl-O-(1-phenyl-2-chloro-vinyl)-phosphate (X). The mixture was heated to 120°C for 8 hrs. After washing with water and drying with anhydrous sodium sulfate, chlobenzene was evaporated under reduced pressure. Fractionation of the residual oil gave 10.0 g (27.4%) of O,O-dimethyl-O-(1-phenyl-2-*p*-nitrophenoxy-vinyl)-phosphate (X) ; b. p. 145～150°C/0.7 mmHg. n_D^{26} 1.5488

Anal. Calcd. for $C_{16}H_{16}O_7NP$; P, 8.5 ; N, 3.82%
Found ; P, 9.0 ; N, 3.66%

O,O-Diethyl-derivative (XIII) was prepared in the same manner as above, from 29.0 g (0.1M) of O,O-diethyl-O-(1-phenyl-2-chloro-vinyl)-phosphate (XI). This was distilled and the fraction boiling at 146～150°C/0.44 mmHg n_D^{28} 1.5303, weighted 15.0g (38.1%)

Anal. Calcd. for $C_{18}H_{20}O_7NP$; P, 7.9 ; N, 3.56%
Found ; P, 8.1 ; N, 3.66%

Likewise as above O,O-Dimethyl-O-(2-chloro-2-*p*-nitrophenoxy-vinyl)-phosphate (XIV) was also prepared from 17.7 g (0.1M) of *p*-nitrophenol potassium salt and 44.1 g (0.2M) of O,O-dimethyl-O-(2, 2′-dichloro-vinyl)-phosphate (D. D. V. P.). This was distilled and the fraction boiling at 155～160°C/0.66 mmHg n_D^{28} 1.5344 weighted 13.7 g (42% from *p*-nitrophenol potassium salt)

Anal. Calcd. for $C_{10}H_{11}O_7ClNP$; P, 9.6 ; N, 4.33 ; Cl, 10.97%
Found ; P, 9.0 ; N, 4.16 ; Cl, 10.63%

The author wishes to express his sincere thanks to Dr. Ooi for the infrared absorption spectrum, Mr. Kadota for observation of the toxicity, Mr. Iwai for performing microanalysis.

The author is also much indebted to Mr. Nakagawa for his earnest experimental assistance.

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6) A. N. Pudovik, *Doklady Akad. Nauk S.S.S.R.*, **105**, 735 (1955); *Chem. Abst.*, **50**, 11230 g (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 266~268, 1960]

Formation of Higher Alcohols in the Fermentation of Threonine by Yeasts

By Masakazu YAMADA and Kiyoshi YOSHIZAWA

The Brewing Experiment Station, Tax Administration Agency

Received May 20, 1959

Several years ago, one of the authors, M. Yamada has found that the fermentation of straight-chain amino acid like alanine or α-amino-n-butyric acid by yeasts gives rise to *iso*-butyl alcohol or active amyl alcohol in contrary to F. Ehrlich's scheme. From this respect we examined the fermentation of *dl*-threonine which one of the hydroxylic amino acids, and obtained an active amyl alcohol as in the case of α-amino-n-butyric acid and it was found that threonine seems to be reduced to the corresponding straight-chain amino acid.

Concerning the alcoholic fermentation of amino acids by yeasts, F. Ehrlich previously proposed the formation of an alcohol which had a carbon chain less one atom than the corresponding amino acid. Although later one of the authors Yamada[1], as well as Thorne[2] and others have studied on this mechanism, the problem still remains unclarified.

Yamada remarked the fact that some amino acids, for instance alanine, were converted to the branched alcohols which had one more carbon atom than the amino acids fermented, in contrast to the scheme proposed by Ehrlich. In this report the alcoholic fermentation of threonine, an amino acid containing a hydroxyl group is described.

The solution of threonine fermented by yeasts became reddish-purple on reaction with vanilline-sulfuric acid reagent showing the formation of fusel oil. This fusel oil was fractionated and analysed. The results evidently showed the formation of active amyl alcohol which had an additional carbon atom than threonine as in the case of the fermentation of α-amino-n-butyric acid.

EXPERIMENTAL

1. Fermemtation and distillation

Two solutions, one containing threonine and the other containing lysine as the nitrogen nutrient, were incubated with the yeast Kyōkai No. 7 at 25°C for 10 days. In case of the threonine solution, fermentation occurred and the fermented solution showed reddish-purple on reaction with the vanilline-sulfuric acid reagent. However, in case of the lysine solution, fermentation hardly occurred [illegible] the same reagent. Four hundred ml of yeast suspension precultured for 3 days was added to 30 l of the threonine solution whose composition is shown in Table I (A). After fermentation at 25°C for 2 weeks the solution was filtered, and the filtrate distilled. Table I (B) shows the [illegible] filtrate gave rise to 1800 ml of 79% ethyl alcohol. This was further fractionated by distillation into 3 fractions as shown in Table II.

The first distillate was redistilled. Five ml of the initial distillate gave a positive Rimini reaction and the p-nitrophenylhydrazine derivative obtained melted at 128°C, thus showing the presence of acetaldehyde.

2. Analysis of the fusel oil

The fusel oil fraction, having some fragrance different from that of isoamyl alcohol, was distilled accurately and divided into 10 parts as shown in Table III. The 3,5-dinitrobenzoate derivative was prepared from each

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[1] M. Yamada, This Bulletin, **8**, 95 (1932).

[2] R. Thorne, *J. Inst. Brewing*, **43**, 288 (1937).

TABLE I. THE COMPOSITION OF MEDIUM BEFORE AND AFTER FERMENTATION

	(A) before fermentation	(B) after fermentation (g/l)
Sugar	90.0	7.95
Threonine	2.68	1.79
K_2HPO_4	1.00	
$MgSO_4 \cdot 6H_2O$	3.00	
pH	5.8	
Alcohol %		4.8
Acid		1.90 ml/10 ml
Yeast (dry)		2.0

TABLE II. THE COMPOSITION OF ALCOHOL FRACTIONS

	quantity	alcohol %	Vanilline-H_2SO_4 reaction
First distillate	150 ml		blue
Ethylalcohol fraction	1350 ml	95	yellow
Fusel oil fraction	17.0 g		reddish purple

TABLE III. THE FRACTIONAL DISTILLATES OF FUSEL OIL FRACTION

No.	temp. at distln. (°C)	quantity (g)	M. Pt. of 3, 5-dinitrobenzoate (°C)
1	79~82	1.5	90.5
2	82~90	1.5	80~84
2′	90~103	1.3	mixture of water and alcohol
3	103~125	1.1	47~49
4	125~128	1.9	74~75
5	128	2.9	76
6	128~130	1.0	74~75
7	130	0.6	58~60
8	130~135	0.3	
9	residue	0.7	—

TABLE IV. THE FRACTIONATED 3, 5-DINITROBENZOATES

No.	number of bands	M. Pt. of the extracts (°C)
1	2 (obscure)	89~90, 90~91
2	2	68, 90~91
3	2	79~81, 61~62
4	2	80~82, 73~75
5	1	76
6	1 (broad)	66~70 (upper), 60~65 (lower)
7	2 (obscure)	68~73, 63~64

fraction and the derivative was chromatographed on a silica gel column according to the method of White and Dryden[3]. The substances fractionated as bands were extracted with ether and crystallized.

Table IV shows the melting points of the fractionated 3,5-dinitrobenzoates. It is apparent that ethyl alcohol occupies a large part of fractions 1 and 2. The main part of the fusel oil consisted of fractions 4,5 and 6. When fraction 4 was chromatographed on a silica gel column, it was divided into 2 parts; the larger part gave crystals melting at 73°C, and the other gave those melting at 80 to 82°C. Fraction 5 the largest fraction of all, gave only a single band after silica gel column chromatography and the melting point of the extracted substance did not vary. The phenylurethan derivative of this substance melted at 31°C. The nitrogen content of this 3,5-dinitrobenzoate was found to be 9.88%. Calculated as $C_{12}H_{14}O_6N_2$; 9.93%. Fraction 6 showed a single broad band by silica gel column chromatography. The substance extracted from its upper part melted at 66 to 70°C, while that extracted from the lower part melted at 60 to 65°C. The nitrogen content of the 3,5-dinitrobenzoate was 10.06%. Fractions 4, 5 and 6 were optically active and the mixed solution of these 3 fractons gave $[\alpha]_D^{15}$ $-5.3°$. From these results it was found that fraction 5 was identical with active amyl alcohol. It was also presumed that the most of fraction 4 should be active amyl alcohol, fraction 6 a mixture of active amyl alcohol and isoamyl alcohol, and the majority of fraction 7 isoamyl alcohol.

It thus becomes clear that the largest constituent of this fusel oil is active amyl alcohol. This result coincided with the result of analysis of the fusel oil produced in the fermentation of α-amino-n-butyric acid which lacks hydroxyl group in contrast to threonine.

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[3] J. White and E. Dryden, *Anal. Chem.*, **20**, 853 (1948).

3. Analysis of acid fraction

The residue of the first distillation was concentrated on the boiling-water bath and alcohol was then added. The precipitate was separated by filtration. Threonine was detected remarkedly on the paper chromatogram of this precipitate. After the removal of alcohol the filtrate was acidified with snlfuric acid and extracted with ether for 72 hrs. Forty g of acid fraction (2.8 meq/g) was obtained as the extract after removing ether. On the paper chromatogram, succinic acid, lactic acid, and traces of fumaric and malic acid were detected. The result of silica gel column chromatography showed that this fraction contained 5.27 g of succinic acid and 1.0 g of lactic acid.

DISCUSSION

The above results evidently shows that the bulk constituent of the fusel oil is active amyl alcohol and this alcohol amounts to 5.8 g if fractions 4, 5 and 6 are assumed to be amyl alcohol. Therefore supposing that, this alcohol is made exclusively from threonine, it can be calculated that 36.8% of threonine consumed is transformed into active amyl alcohol. Other alcohols except ethyl alcohol are rarely produced. Thus it may be concluded that active amyl alcohol produced as the characteristic product of alcoholic fermentation of a medium containing threonine as the nitrogen source. This result coincides well with the result of experiments conducted on alcoholic fermentation of α-amino-n-butyric acid by yeasts which one of the authors Yamada[4], previously examined. The presence of the hydroxyl group in threonine had no effect on the alcohol produced. The mechanism of this transformation is still entirely unknown. A trace of isoamyl alcohol produced was concieved to have been made from leucine which originated from yeast protein as in the case of ordinary alcoholic fermentation by yeasts previously reported[5]. Conclusively it should be noted that a large quantity of succinic acid is produced as the same time in the fermentation of threonine.

Acknowledgments The authors wish to express their thanks to Tanabe Seiyaku Co., Ltd., Osaka for their kindly supplying the precious samples used here.

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4) M. Yamada, This Bulletin, **8**, 95 (1932).
5) M. Yamada, K. Yoshizawa and S. Sasaki, This Bulletin, **22**, 314 (1958).

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Respiration and Carbohydrate Metabolism of Potato Tuber. Part I

By Koichiro HONDA and Yoshiaki ODA

Department of Agricultural Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, near Osaka

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The activity of tyrosinase, isolated from potatoes is suppressed in the presence of redox dyes and the composed dehydrogenase systems. The oxygen uptake of potato tuber increases, but after being cut into slices the dehydrogenase activity of tuber decreases in the lapse of time. This increase in oxygen uptake is caused by tyrosinase. Accordingly, tyrosinase does not seem to play the role as a terminal oxidase in the electron transferring systems.

INTRODUCTION

The oxygen uptake of plant tissues has been found to be increased when they are injured or infected with pathogens. The potato tuber (*Solanum tuberosum*, L) is of no exception and an increase in oxygen uptake is observed when it is cut into slices. A number of papers reporting on this phenomenon have been published[1~3]. In the previous papers the author[4] has reported as follows: 1) The oxygen uptake of potato slices gradually increases, but the activity to reduce methylene blue decreases when the slices are kept in running water. 2) The oxygen uptake of fresh slices is inhibited by monoiodoacetate, and not by thiourea while that of the slices kept in running water (treated slices) is inhibited by thiourea and diethyldithiocarbamate. 3) The oxygen uptake of fresh slices is increased by the addition of *p*-cresol while this is not the case with the treated slices which have already exhibited an increased oxygen uptake. These facts suggest that different terminal oxidase systems contribute to the oxygen uptake in treated and fresh slices. 4) The effects of various redox dyes on the oxygen uptake of the treated slices and on the tyrosinase dopa system isolated from potato tuber are very similar, this fact suggesting that tyrosinase might play the main role in the increased oxygen uptake of the treated slices.

In the present research, in order to investigate that enzymatic mechanism of decrease in activity of reducing methylene blue which accompanies the increased tyrosinase activity in the treated potato slices, some experiments were carried out with enzymes and the soluble part of potato juice.

METHODS AND MATERIALS

1. Reducing activity of potato juice. Reducing activity of potato juice was estimated at 25°C by measuring the time required to decolorize methylene blue in a system consisting of 2.5 ml of potato juice, 2.0 ml of 0.1 M phosphate buffer, pH 7.2 and 0.1 ml of 0.01% methylene blue solution.

Furthermore, the activity was also estimated by measuring the intensity of the red color caused by the reduction of triphenyl tetrazolium chloride (TTC). Two and a half ml of potato juice was incubated with 0.1 ml of 0.01% methylene blue solution, 1.0 ml of 1×10^{-3} M TTC solution, and 1.0 ml of 0.1 M phosphate buffer, pH 7.2 at 25°C for 120 minutes in vacuo. The reaction was stopped by the addition of 2.0 ml of 10% trichlo-

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[1] E. G. Barron, *Arch. Biochem.*, **28**, 377 (1950).
[2] A. Schade, *Arch. Biochem.*, **20**, 211 (1949).
[3] K. Thimann, *Arch. Biochem. and Biophys.*, **53**, 239 (1954).
[4] K. Honda, *J. Agr. Chem. Soc. Japan* (Nippon Nogeikagaku Kaishi), **29**, 32 (1955).

roacetic acid and then the formazan produced was extracted with 10 ml of ethyl acetate. The intensity of red color was estimated photometrically at 485 mμ.

2. Tyrosinase activity. Tyrosinase activity was determined by two different methods. 1) Oxygen uptake was measured manometrically by using a Warburg apparatus at 30°C. 2) Quinone, formed from catechol by the action of tyrosinase, was measured according to Dawson's chronometric method[5].

3. Alcohol dehydrogenase. The enzyme was prepared from dried Fleischmanns baker's yeast according to the method of Racker[6].

4. Tyrosinase. The enzyme, prepared from potato tuber according to Kubowitz's method[7], was used after being dissolved in 0.03 M phosphate buffer, pH 7.3.

5. Mitochondria. Fresh rat liver (6 g) was rapidly homogenized in a glass homogenizer with 10 parts of 0.9% potassium chloride solution, and the brei was centrifuged for 10 minutes at 600 × g. The supernatant was centrifuged for 20 minutes at 8000 × g and the mitochondrial sediment, after being washed in the original volume of potassium chloride solution, was suspended in 10 ml of potassium chloride solution. These procedures were carried out in the cold. The preparation was carried out just before use.

6. Glucose-6-phosphate (G-6-P). This phosphate was prepared from soluble starch by the combined action of potato phosphorylase and broad bean phosphoglucomutase according to the method of Inoue[8].

7. Triphosphopyridine nucleotide (TPN). The TPN was obtained from hog liver as a crude preparation following the method of Okunuki[9]

8. Diphosphopyridine nucleotide (DPN). The DPN was supplied through the courtesy of Dr. K. Okunuki, Prof. of Osaka University.

RESULTS AND DISCUSSION

The first experiment was conducted to find whether potato juice would give results similar to those of potato slice or not. The juice was prepared by homogenizing the slices (fresh or treated) at 0°C, followed by centrifuging at 1000 × g to remove starch, cell walls, etc. Thimann[3] has reported that the slices which were kept at 30°C for twenty-four hours gave the results similar to those obtained with the slices kept in running water for 72 hours. Therefore the juice separated from the slices kept at 30°C was also investigated.

As can be seen in table I, both juices obtained from the slices kept in running water and the juice obtained at 30° C showed an increase in oxygen uptake and a decrease in reducing activity. The latter activity was not restored even by the addition of DPN, suggesting the inactivation of dehydrogenase systems in the potato juice.

In the previous paper[4], it was reported that in the presence of redox dyes, the oxygen uptake is suppressed by a tyrosinase system and by the treated slices; the lower the potential of the dyes the greater the suppression. To study the relationship between the fact mentioned above and the fact that the juice which has a decreased reducing activity shows an increased oxygen

TABLE I. O_2-UPTAKE AND REDUCING ACTIVITY OF POTATO JUICE

	O_2-Uptake during 30 min.		Reducing Activity	
	mm^3/cc	$mm^3/N\,mg$	reduction of Methylene Blue (min.)	reduction of TTC (optical d.)
Juice from fresh slices	28.0	9.5	8	0.468
〃 〃 + DPN*	—	—	7	0.470
Juice from slices maintained at 30°C for 20 hours	40.0	11.7	> 60	0.036
〃 〃 + DPN*	—	—	> 60	0.082
Juice from slices maintained in running tap water for 72 hours	33.0	12.0	> 60	0.000
〃 〃 + DPN*	—	—	> 60	0.000

* 10γ of DPN was added.

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[5] C. Dawson, *J. Am. Chem. Soc.*, **66**, 514 (1944).

[6] E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).

[7] F. Kubowitz, *Biochem. Z.* **292**, 221 (1937).

[8] Y. Inoue, K. Onodera and T. Ito, *J. Agr. Chem. Soc. Japan* (Nippon Nogei-kagaku Kaishi), **30**, 59 (1956).

[9] K. Okunuki, *Koso Kenkyuho* (Methods in Enzymes) Asakura Shoten, Tokyo vol. **1**, 682 (1955).

TABLE II. DEHYDROGENASE-SYSTEM AND TYROSINASE ACTIVITY

Incubation Medium	O_2-Uptake mm^3 (during 15 min.)
tyrosinase + catechol	79.0
tyrosinase + catechol + alc.	78.0
tyrosinase + catechol + alc. + DPN	81.0
tyrosinase + catechol + alc. + DPN + alc. dehydrogenase	73.0
〃 + 〃 + 〃 + 〃 + 〃 (2)	66.0
〃 + 〃 + 〃 + 〃 + 〃 (3)	58.0
〃 + 〃 + 〃 + 〃 + 〃 (5)	56.0

The incubation medium contained: 50 m moles of catechol, 50 m moles of alcohol, 5 mg of DPN, 160 units* of alcohol dehydrogenase, and tyrosinase in 3 ml of 0.03 M phosphate buffer, pH 7.2

(2) 360 units of alcohol dehydrogenase
(3) 480 units of alcohol dehydrogenase
(5) 800 units of alcohol dehydrogenase

* The activity of alcohol dehydrogenase is defined according to Racker6).

TABLE III. DEHYDROGENASE-SYSTEM AND TYROSINASE ACTIVITY

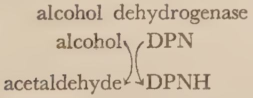
Incubation Medium	Quinone formed mg/min.
tyrosinase + catechol	3.75
tyrosinase + catechol + alc. dehydrogenase system	2.44
tyrosinase + catechol + mitochondria + α-ketoglutarate	2.83
tyrosinase + catechol + KCl	3.65

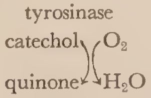
The incubation medium contained: 3 mg of ascorbate, 50 mg of catechol, and alcohol dehydrogenase system, or in place of the last, mitochondria from 0.6 g of wet rat liver plus 5 m moles of α-ketoglutarate, in 100 ml of 0.02 M citrate-phosphate buffer, pH 7.2.

"Alcohol dehydrogenase system" consisted of 750 units of alcohol dehydrogenase, 5 mg of DPN and 500 m moles of alcohol.

KCl solution was added in a same volume as the mitochondria suspension.

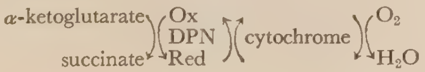
uptake as shown in table I, the oxygen uptake of the tyrosinase-catechol system was measured in the presence of an alcohol dehydrogenase system in place of the redox dye.





As it is seen in table II, the oxygen uptake decreased along with the increase in concentration of alcohol dehydrogenase, showing that there is some competition between these two systems.

A similar phenomenon was also observed when the following system was used in place of the alcohol dehydrogenase system (see table III).



It is reported10) that tyrosinase is inhibited in the presence of chloride ions. However, no inhibition was observed at the concentration of chloride ions which were used to suspend the mitochondria.

From the results mentioned above it may be confirmed that when the slices are kept in running water or at 30°C, the dehydrogenase activity decreases and the tyrosinase activity which had been suppressed by the dehydrogenase system, increases to play the main role in the oxygen uptake of the juice.

The fact that tyrosinase activity is suppressed in the presence of a dehydrogenase system or a redox dye may be interpreted as follows: the activity of tyrosinase may be due to the reversible reaction occurring between cupric and cuprous

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10) A. B. Lerner, *Arch. Biochem. and Biophys.*, **36**, 473 (1952).

TABLE IV. DEHYDROGENASE ACTIVITY LINKED TO DPN AND TPN

	Incubation medium				Reduction of TTC (optical d.)
	G-6-P	Mg	TPN	DPN	
Dialyzed Soluble Fraction	+	+	+	−	0.144
	+	−	+	+	0.088
	+	+	−	+	0.009
	+	+	−	++	0.009
Soluble Fraction	−	+	+	−	0.033
	−	+	−	+	0.009

Incubation medium: 200 μ moles of G-6-P, 3 μ moles of $MgCl_2$, 10 γ of DPN, 100 γ of TPN, 2 μ moles of adenosine triphosphate, 0.4 μ moles of TTC and 0.1 mg of methylene blue were dissolved in 2.0 ml of 0.1 M phosphate buffer, pH 7.2 and the mixture was incubated with 2.0 ml of the soluble part of fresh slices. Incubated at 30°C for 60 min. in vacuo. (++) means twice as much of DPN.

TABLE V. CHANGES IN DEHYDROGENASE ACTIVITIES AFTER CUTTING INTO SLICES

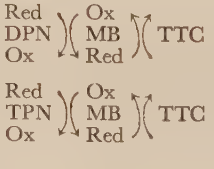
	activity linked to TPN (Reduction of TTC during 30 min. optical d.)	activity linked to DPN (Lactate formed during 30 min. μ moles)
soluble part from fresh slices	0.085 0.002*	0.6 0.0*
soluble part from treated slices	0.392	0.2

The soluble part of potato slices (2.0 ml) was incubated at 30°C for 30 minutes in vacuo with 2.0 ml of 0.1 M phosphate buffer pH 7.2, containing 200 μ moles G-6-P, 10 γ of DPN, or 100 γ of TPN, 3 μ moles of magnesium chloride, 0.4 μ moles of TTC and 0.1 mg of methylene blue. After deproteinization by 10% trichloroacetic acid, lactate was determined by the method of Barker and Summerson[15].

* Without G-6-P

ions at the active center of the enzyme. The addittion of a redox system to tyrosinase may cause this reaction to tend to the cuprous side, and thus it may inactivate the enzyme.

From the results shown in table I, it was deduced that the decrease in the reducing activity is caused by the inactivation of some dehydrogenase systems in the juice during treatment. A number of reports on the presence of dehydrogenases related to the metabolic pathway in the potato tubers have been published[1,11~14]. In order to study what kind of dehydrogenase is inactivated during the treatment, the following experiment was conducted. As it is well known, the enzymes of glycolysis and G-6-P dehydrogenase are contained in cytoplasmic matrix (fluid). The soluble part was obtained by centrifuging the juice from fresh slices at 3×10^4 g at 0°C, and by dialyzing in an ice box for six hours against several changes of 0.1 M phosphate buffer, pH 7.2. The dehydrogenase activities of the soluble part linked to DPN and TPN were determined by measuring the color intensities of formazan produced as shown in the following table:



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[11] D. Anderson, *Plant Physiol.*, **27**, 675 (1952).

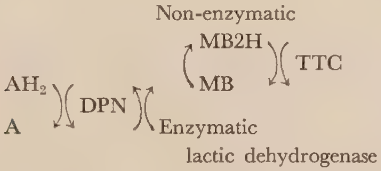
[12] J. Bonner, *Plant Physiol.*, **24**, 720 (1949).

[13] B. Axelrod, *J. Biol. Chem.*, **204**, 939 (1953).

[14] S. Schwimmer, *J. Agr. and Food Chem.*, **1**, 1063 (1953).

[15] J. Barker and W. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).

As it is shown in table IV the dehydrogenase activity linked to TPN could be detected by this method, but the activity linked to DPN was scarcely detected as in case of the juice (containing mitochondria). This may be attributed to the following reason; in the absence of mitochondria in the incubation medium, the reaction may be changed to the formation of lactic acid. Formazan may not be produced, because of the weakness of the nonenzymatic reoxidation of DPNH by methylene blue as compared to the reoxidation by lactic dehydrogenase.



Therefore after the addition of G-6-P to the soluble part, lactic acid formed from G-6-P was determined as a measure of the glycolytic activity in place of dehydrogenase activity linked to DPN.

As shown in table V, the glycolytic activity of the soluble part obtained from treated slices decreased, suggesting the inactivation of dehydrogenases involved in these processes, but the dehydrogenase activity linked to TPN showed an increase.

From these results obtained by using the enzyme systems and the soluble part of potato juice, it might be concluded as follows. When the potato slices are maintained in running water or maintained at 30°C, the glycolytic activity is inactivated, and the tyrosinase activity which had been suppressed by the glycolytic activity—mainly dehydrogenase activity in this pathway—increases and comes to play the main role in the oxygen uptake. The rise of the dehydrogenase activity linked to TPN will be reported in our next paper.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 274~277, 1960]

Combustion Temperatures of Some Unblended Cigarettes and the Transfer of Nicotine into Cigarette Smoke*

By Yūsuke KOBASHI, Sōichi SAKAGUCHI and
Masao IZAWA

Tobacco Smoking Research Laboratory, Central Research Institute,
Japan Monopoly Corporation

Received June 13, 1959

The combustion-zone temperature of cigarettes made from each individual tobacco leaf of several varieties, was measured as follows: Turkish—846°C, Bright—844°C, Japanese Native "Nambu" —832°C, Japanese native "Matsukawa", "Daruma" and Burley—812°C. Within the range of 1.0 and 1.2 g for Turkish and Bright, and 0.7 and 0.9 g for the others, it was found that throughout the varieties, thickness of the cigarette has no affect on the combustion-zone temperatures. The amount of nicotine contained in the smoke (Y mg) per one gram of the consumed cigarette is mainly affected by the contents of nicotine in the cut leaves (X %), and the relation between both values of each variety of tobacco leaves is shown as follows: $Y=1.7\,X$.

Till quite recently, several papers dealing with the chemical components of cigarette smoke[1~4] have appeared, and the present authors[5] have also provided some information concerning this problem. From the standpoint of the improving smoking taste and the reduction of nicotine in the smoke, it seems desirable to make clear the combustion mechanism of tobacco, especially, the thermal decomposition and transfer phenomenon of tobacco alkaloids.

Studies conducted in this laboratory[6,7] indicated that the combustion temperatures of cigarettes lie within the range from 794°C, to 827°C, and the temperatures are not significantly affected by smoking conditions or the moisture content of cigarettes.

Because of its physiological importance, many workers have eagerly studied on the problem of nicotine transfer into tobacco smoke. Nagy[8] reported that the total quantity of nicotine contained in the leaf comes into the smoke. His opinion, however, is considered to be inappropriate. Lehman[9] has obtained a transfer rate of 80.2~98.7%. Pyriki[10] reported that 21.0~31.0% of nicotine in the leaf enters into the cigarette smoke. In Consumer Reports[11], it has been reported that the amount of nicotine contained in the smoke of 37 brands of cigarettes is within the range of from 0.4 mg to 4.0 mg per cigarette.

In the previous papers[6,7], it was demonstrated that the amount of nicotine transferred into the smoke is altered by smoking procedures, and increases from 13.7% to 38.5% in proportion

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* Studies on the thermal decomposition of tobacco alkaloids, part III.

[1] M. E. Hobbs, *Science*, **124**, 108 (1956); *Anal. Chem.*, **28**, 211, 2002 (1956); **29**, 106 (1957); **30**. 547, 1401 (1958); *Nature*, **182**. 865 (1958).

[2] G. P. Touey, *Anal. Chem.*, **27**, 1685, 1788 (1956).

[3] H. R. Hanmer, *Ind. Eng. Chem.*, **29**, 45 (1937); *Anal. Chem*, **25**, 1418 (1953).

[4] J. D. Mold, *Tobacco Science*, **1**, 38, 40, 161 (1957).

[5] M. Izawa, Y. Kobashi, This Bulletin, **21**, 357, 364 (1957); **22**, 47 (1958); **23**, 194, 198 (1959).

[6] Y. Kobashi, S. Sakaguchi and M. Izawa, *ibid.*, **23**, 528 (1959).

[7] Y. Kobashi, S. Sakaguchi and M. Izawa, *ibid.*, **23**, 532 (1959).

[8] L. Nagy, *Biochem. Zeitschrift*, **254**, 94 (1932).

[9] K. Lehman, *Arch. f. Hyg.*, **68**, 321 (1908).

[10] C. Pyriki, *Ber. des Inst. f. Tabak Forschung*, Heft **1**, 62 (1954).

[11] Consumer Reports, Feb., 1955.

TABLE I. WEIGHT AND MOISTURE CONTENT OF SAMPLE CIGARETTES

		Turkish	Bright		Japanese Native			Burley
			Japanese	U.S.A.	Nambu	Matsukawa	Daruma	
Weight (g)	Thin cigt.	1.057	1.053	1.071	0.711	0.708	0.655	0.707
	Thick cigt.	1.266	1.255	1.284	0.907	0.914	0.795	0.922
Moisture (%)		13.74	14.40	16.20	13.65	11.80	12.40	12.44

TABLE II. COMBUSTION-ZONE TEMPERATURE (°C) OF CIGARETTES

	Turkish	Bright		Japanese Native			Burley
		Japanese	U.S.A.	Nambu	Matsukawa	Daruma	
Thin cigt.	844.6±13.3	840.3±15.0	840.7±12.1	828.7±14.8	810.3±8.8	815.0±13.8	817.7±10.9
Thick cigt.	847.0±13.0	850.3±12.3	840.7±11.1	855.3± 9.5	817.3±7.5	806.0± 7.4	807.3±11.9
Average	845.8± 9.0	844.3±6.2		832.0± 8.4	812.2±4.7		812.5± 7.9

Every value was calculated from 30 experiments.
The confidence limits are accepted at the 5% level of significance.

with the increase in puff velocities. However, influences of the moisture content are out of question. The present paper deals with combustion temperature, combustibility of several unblended cigarettes, effect of the amount of cut leaves filled in cigarettes on their physical properties, and the transfer of nicotine into cigarette smoke.

EXPERIMENTAL

Preparation of Unblended Cigarettes: Each unblended cigarette having a standard length of 70 mm, used in this investigation, was made from Turkish, flue-cured (Japanese Bright and U. S. Bright), sun-cured (Japanese Native "Matsukawa" and "Daruma"), Burley and fire-cured (Japanese Native "Nambu") tobacco leaves, respectively. After being conditioned for three weeks in an atmosphere of 20°C and 60% relative humidity, each of the seven varieties of cigarettes was segregated into two groups of thickness, corresponding to "thin" 1.0g and "thick" 1.2g for Turkish and Bright, and 0.7g and 0.9g for Japanese Native and Burley. The weight and moisture content of the samples are shown in Table I. Each unblended cigarette was smoked up to the length of 45 mm from the lighted-end in a room maintained at 20°C and 60% relative humidity.

Apparatus and Procedures: Smoking apparatus, smoking procedures and the measurement of combustion-zone temperature were essentially the same as described previously[6]. Thirty readings of combustion-zone temperatures were made for each kind of unblended cigarettes and the true temperatures were calculated at the confidence level of 95%. In order to compare the combustibility of the cigarettes, puff numbers corresponding to one gram of cut leaf consumption in the cigarette were measured and average values of twenty experiments were calculated. The amount of nicotine in the leaf and in the smoke was determined by the method of Willits[12] and each value reported here is the average of ten experiments. The pH values of the leaf and the smoke were measured as follows: An aqueous solution of 20 ml, prepared by extraction from two grams of leaf samples for 15 hours, was subjected to measuarments employing a glass electrode pH meter. In the case of cigarette smoke, two cigarettes were smoked according to the same technique described previously[6]. However, in this experiment a glass tube filled with cotton which was used for absorption of nicotine in the smoke was not employed. The produced smoke was caught into 50 ml of water in an absorbing bottle (See reference (6) Fig. 1 (D)), and after the smoke solution was subjected to pH measurement, the average values of five experiments were calculated.

RESULTS AND DISCUSSION

Combustion-Zone Temperature of Cigarettes: The combustion-zone temperatures obtained from each sample are shown in Table II at the confidence level of 95%. No significant differences were observed in combustion-zone temperatures between thinly and thickly filled cigarettes as

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12) C. O. Willits, *Anal. Chem.*, **22**, 430 (1950).

TABLE III. CIGARETTE COMBUSTION AND NICOTINE IN SMOKE OF VARIOUS TOBACCOS

		Turkish	Bright		Japanese Native			Burley
			Japanese	U.S.A.	Nambu	Matsukawa	Daruma	
Nicotine in Leaf (%)		1.05	4.07*	2.72	2.50	1.76	1.92	1.44
Nicotine in Smoke (mg)	Thin cigt.	0.97	3.81	2.52	1.70	1.18	1.34	0.95
	Thick cigt.	1.13	4.41	2.88	2.05	1.40	1.56	1.14
Nicotine in Smoke per One gram of Smoked Portion of Cigarette	Thin cigt.	1.73	6.88	4.51	4.60	3.14	3.90	2.55
	Thick cigt.	1.63	6.63	4.30	4.30	2.68	3.69	2.21
Transfer Rate (%)**	Thin cigt.	16.47	16.87	16.58	18.40	17.79	20.27	17.66
	Thick cigt.	15.87	16.25	15.79	17.11	15.97	19.18	15.81
Puff Number	Thin cigt.	18.12	16.1	14.7	7.9	6.5	6.2	6.3
	Thick cigt.	22.5	19.5	18.2	9.9	9.6	7.8	9.2
Puff Number per One gram of Smoked Portion of Cigarette	Thin cigt.	32.4	29.1	26.4	21.3	17.3	18.0	16.9
	Thick cigt.	33.2	29.3	27.3	20.6	19.4	18.4	15.7
pH of Leaf		5.2	5.1	4.7	5.6	6.1	6.4	6.2
pH of Smoke	Thin cigt.	5.9	5.0	5.3	5.9	6.0	5.3	5.8
	Thick cigt.	5.9	5.4	5.1	6.0	6.0	5.5	5.9

* Because the Japanese Bright was selected from the most heavily bodied leaf, its nicotine content is rather higher.

** Nicotine in smoke/Nicotine in consumed cigarette.

far as the same variety is concerned. When the combustion-zone temperatures of Turkish, Bright, “Nambu”, “Matsukawa”, “Daruma” and Burley are compared each other by the application of stochastics, it was evidently shown that no significant differences exist between Turkish and Bright of which values are the highest among all varieties. Although the combustion-zone temperature of “Nambu” is lower than that of Bright and Turkish, it is higher than other varieties such as “Matsukawa”, “Daruma” and Burley. The values of Native (“Matsukawa” and “Daruma”) do not differ from that of Burley, and both values are the lowest among the five varieties. The following series of combustion-zone temperatures were obtained by means of testing mean value differences: Turkish (846°C), Bright (844°C),>“Nambu” (832°C),> Burley (812°C), and Native (“Matsukawa” and “Daruma”) (812°C).

Combustibility of Cigarettes: In order to compare the combustibilty of each cigarette, the puff numbers per one gram of consumed cigarettes were calculated and are tabulated in Table III. From the table it is evident that, within the same variety, differences of these puff numbers between thinly and thickly filled cigarettes are very small and the amount of cut leaves filled in one cigarette does not exert any influence on the combustibility. However, the variations among different varieties are evidently represented. Turkish-tobacco cigarettes are consumed most slowly, and the combustibilities rise in the order of Bright, “Nambu”, “Matsukawa”, “Daruma” and Burley. This evidence may illustrate that the Turkish- or Bright-cigarettes contain a larger amount of high polymers such as carbohydrates or resins, which are considered to have relation to combustibility, then it takes more time for combustion of the same weight of the cigarettes than thase of Native or Bright-cigarettes poor in those substances. An almost similar trend has also been observed on the combustion-zone temperature.

Effect of Nicotine Content and Weight of Cigarettes on Nicotine Transfer into Smoke: When the cigarettes were smoked up to a length of 45 mm from the lighted-end, most of the nicotine transfer rates, with only one exception of “Daruma” variety, ranged between 15.8% and 18.4%, as shown in Table III. On the contrary, the amount of nicotine transferred into smoke varies according to the cigarette variety and nicotine content of the original leaves, and ranges from

0.9 mg to 4.41 mg per cigarette. However, for accurate comparison, it is necessary to discuss the amount of nicotine transferred into the smoke of one gram of the consumed portion of cigarette, because the amount of cut leaves filled in one cigarette sample differs according to the variety. As shown in Table III, it is evidently recognized that the amount of nicotine transferred into cigarette smoke per consumed cigarette is markedly affected by the quantity of nicotine contained in the leaves. The relation between the nicotine content in the leaves and the amount of transferred nicotine into smoke is expressed by the following equation:

$$Y=1.7\,X$$

X=Nicotine content of leaves (%)

Y=Amount of nicotine entering into smoke per one gram of consumed cigarette (mg)

pH Values of Leat and Smoke: As shown in Table III, among the cigarette varieties, little difference was observed in pH values of the leaf and smoke. The pH values of smoke of Bright, Turkish and "Nambu" varieties are higher than those of the leaves. On the contrary, the pH values of smoke of Burley and Native are lower than values of the leaves. However, each pH value showed that reactions are inclind to the acidic side, and in this experiment, no relationship was found between pH values and transfer rates of nicotine into the smoke.

Therefore the following conclusion is derived from the above mentioned results:

1) Although the combustion temperature somewhat varies according to the cigarette varieties, it is considered that there is no relation between the combustion-zone temperature and the quantity of nicotine in the smoke.

2) The combustibility of cigarettes varies extremely, and both pH values of smoke and leaves slightly altered in accordance with the variety of tobacco leaves. However, combustibilities and pH values are not important factors for the nicotine transfer into smoke.

3) In regard of the same variety, the variation of the "transfer rate" of nicotine into smoke is very small. However, the "quantity" of nicotine in smoke alteres remarkably according to the variety of the tobacco leaf and mainly increases with the increase in the amount of nicotine contained in the leaves.

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Transglucosidation Action of Crystalline Mold Maltase

Part I. Transfer of Glucose from α-Heteroside to Alcohols and Sugars

By Shiro SUGAWARA, Yukihiko NAKAMURA and
Tokuji SHIMOMURA

Department of Agricultural Chemistry, University of Hokkaido, Sapporo

Received August 3, 1959

The transglucosidation action of crystalline mold maltase was investigated. Glucosyl residue of *p*-nitrophenol-α-D-glucoside was transferred to the acceptors, alkyl- and arylalcohols, and α-ethylglucoside was isolated as the transglucosidation product. The degree of glucosyl transfer was 60~70% and no selection among acceptors was observed. The enzyme had no synthetic action of α-glucoside from free glucose and alcohol. Glucosyl residue of the donor was also transferred to sugar acceptors, and isomaltose and other unknown oligosaccharides were detected by paper-chromatography.

The authors have succeeded in the crystallization of maltase from "koji" mold[1] and Taka-diastase[2]. This enzyme hydrolyzed maltose and phenol-α-glucoside, but not appreciably methyl-α-glucoside[1]. The attitude to these substrates was identical with that of crystalline transglucosidase isolated from *Asp. niger* by Tujisaka and Fukumoto[3].

On the other hand, in many glucosidases it has been found that the enzymes act on the substrate in the presence of acceptors to transfer the glucosyl residue of substrate to these acceptors[4]. Accordingly, hydrolysis and transglucosidation action may be interpreted in terms of acceptor difference.

From such point of view it necessitated an investigation of transglucosidation action of our crystalline mold maltase. The experimental results showed that the enzyme acts on *p*-nitrophenol-α-D-glucoside (PNPG) or phenol-α-D-glucoside to transfer the glucosyl residue to several alkyl- and arylalcohols, and sugars. These findings and the results of experiments on transglucosidation action on maltose which will be reported in the following paper showed that the crystalline maltase of mold to be a transglucosidase, identical with the crystalline transglucosidase isolated by Tujisaka and Fukumoto.

EXPERIMENTAL

Materials and Methords

Crystalline maltase was isolated from "koji" by the authors' method[1]. *p*-Nitrophenol liberated from the donor, PNPG, was determined electro-photometrically at 410 mμ and glucose liberated reductometrically according to the method of Somogyi.

The degree of glucosyl transfer is expressed as follows:

$$\text{degree of glucosyl transfer: } (\%) = \frac{[p\text{-nitrophenol, liberated}] - [\text{glucose, liberated}]}{[p\text{-nitrophenol, liberated}]} \times 100$$

Glucosyl transfer from PNPG to several Alkyl- and Arylalcohols.

For a quantitative test, PNPG was used as the glucosyl donor and methanol, ethanol, isopropanol, *n*-butanol, ethyleneglycol, phenol and *p*-cresol as acceptors.

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[1] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **23**, 156 (1959).

[2] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **24**, 112 (1960).

[3] Y. Tujisaka and J. Fukumoto, *Symp. on Enz. Chem. Japan*, **10**, 84 (1958).

[4] c.f. T. Miwa, *Koso Kenkyuho*, **2**. 580 (1957).

Conditions of the reaction were as follows: PNPG, M/300; acceptor, M/150; acetate buffer (pH 5.0), M/100; enzyme solution (0.5%), 0.5 ml in 10 ml of reaction mixture. The reaction mixture was incubated at 40°C for two hours. After incubation liberated glucose and PNP were determined, and the degree of transfer was calculated by the equation described above.

The results (Table I) indicated that the action of glucosyl transfer occurred. No selection among the acceptors was found in these experiments and the degree of transfer was almost 60~70%. In case of the absence of the acceptor the amount of PNP liberated was higher than that of glucose liberated and, this also indicates that transglucosidation action has occurred. In this case, some parts of glucose liberated act as an acceptor of glucosyl residue of PNPG to produce disaccharide, isomaltose. This will be discribed in a later section.

TABLE I. TRANSFER OF GLUCOSE FROM PNPG TO ALKYL- AND ARYLALCOHOLS BY THE ACTION OF CRYSTALLINE MOLD MALTASE

Acceptor	Glucose liberated %	PNP liberated %	degree of transfer %
none	8.88	8.95	0.8
methanol	3.60	9.58	62.4
ethanol	5.85	20.19	71.0
isopropanol	4.80	19.05	74.7
n-butanol	4.05	10.65	61.9
ethyleneglycol	2.25	10.95	79.5
phenol	2.50	9.78	74.4
p-cresol	2.85	9.41	69.7

The condition of reaction is described in the text.

Isolation of α-Ethylglucoside formed by Enzymatic Transfer of Glucose.

Experiments on the isolation of the transglucosidation product was performed to demonstrate direct evidence for enzyme action. In this experiment phenol-α-glucoside was used as the donor and ethanol as the acceptor. Two g of phenol-α-glucoside was dissolved in 100 ml of water and added 5 ml of ethanol. Five ml of the enzyme solution was added to the mixture and pH was adjusted to 4.0 with 1 N-acetic acid. The reaction mixture was incubated at 40°C for thirty hours and during this period phenol-α-glucoside was completely splitted. The solution was subsquently heated in a boiling water bath for ten min. and filtered. The fiiltrate was shaken with ether repeatedly to remove phenol and then, glucose was removed from the subsequent solution with bakers' yeast. After centrifugation, the supernatant was heated in a boiling-water bath and filtered. The filtrate was concentrated to dryness under diminished pressure and extracted with 20 ml of hot ethanol. Ethanol was evaporated to dryness on a water bath and the residue was extracted with 5 ml of boiling water and the extract was cooled. Ethyl-α-glucoside obtained was recrystallized from water. Yield ca. 50 mg, m. p. 111~114°C. Published value. 113~114°.

Evidence for Absence of Synthetic Reaction from Glucose and Alcohol by the Enzyme Action.

Although the results described above present evidence for the glucosyl transfer, there still remains the question whether the synthetic reaction of glucoside, not the glucosyl transfer, with alcohol used as the acceptor and glucose liberated by the enzyme action had taken place or not.

TABLE II. EVIDENCE FOR THE ABSENCE OF SYNTHESIS OF GLUCOSIDE FROM FREE GLUCOSE AND ALCOHOL BY ENZYME ACTION

	Time of incubation	Glucose determined
pH 3	3 hrs.	10.2 mg
	15	10.1
	24	10.1
pH 5	3	10.2
	15	10.5
	24	10.3
pH 7	3	10.1
	15	9.9
	24	10.3

Details are described in the text.

An attempt to resolve this question was made in a solution of glucose and methanol. One ml of 5% glucose solution, 0.25 ml of 0.5% enzyme sol., and 0.5 ml of M/15 citrate buffer were mixed and made up to 4 ml with water. To this sol., 1 ml of methanol was added and incubated at 37°C. No decrease of glucose was observed after twenty-four hrs incubation in the range of pH 3~7 tested, indicating that the synthesis of glucoside had not taken place (Table II).

Transfer of Glucose from PNPG to Glucose, Fructose and Mannose.

In hydrolysis of PNPG in the absence of the acceptor alcohol, as described in the preceding section, the amount of PNP liberated was higher than that of glucose liberated. This observation is to be interpreted as a result of transglucosidation action and subsequent formation of oligosaccharide.

Thus, in order to demonstrate the oligosaccharide formation, experiments were conducted using glucose, fructose and mannose as acceptors and the product was checked by paperchromatography.

Thirty mg of PNPG was dissolved in 5 ml of water and added 0.5 ml of 0.5% enzyme sol., 0.5 ml of M/15 cetrate buffer (pH 4.0) and 1 ml of 5% glucose sol.. The reaction mixture was incubated at 30°C and reducing power was estimated at intervals. The decreace in reducing power (calculated as glucose) after fifteen hrs was 3% and 14% after twenty-four hrs. Glucose and were detected by paperchromatography after twenty-four hrs'. incubation.

The same experiments were conducted using fructose or mannose as glucosyl acceptors. Paperchromatography showed the formation of certain oligosaccharides by the detection of three unknown spots as well as spots of the monosaccharides and isomaltose using fructose as the acceptor. However in case of mannose thers was only one spot assumed to be a transglucosidation product other than isomaltose (Fig. 1).

Studies of isolation and identification of these products is now in progress.

The detection of isomaltose by paperchromatography in this experiment leads us to the conclusion that glucosyl residue of substrate is transferred to the 6 position of the acceptor glucose, and this has been further confirmed by isolation and identification of many oligosaccharides possessing α-1,6 glucosidic linkages produced by the action of this enzyme on maltose, which will be reported in the following paper.

SUMMARY

1. Glucosyl transfer from *p*-nitrophenol-α-D-glucoside to several alkyl- and arylalcohols was observed with crystalline mold maltase. α-Ethylglucoside was isolated as a transglucosidation product, indicating the evidence of such action.
2. No reaction of α-glucoside synthesis from free glucose and alcohol could be observed with mold maltase.
3. By using glucose, fructose or mannose as glucosyl acceptors, the glucosyl residue of *p*-nitrophenol-α-D-glucoside was transferred to these acceptor sugars. Paperchromatography showed the formation of isomaltose from glucose, three unknown oligosaccharides from fructose and one unidentified oligosaccharide from mannose, respectively.

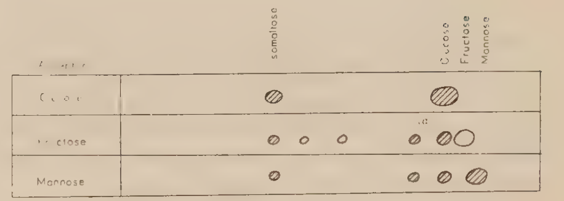

FIG. 1. Paperchromatogram of Sugars produced from PNPG and Acceptor Sugars by Crystalline Mold Maltase

Solvent: Butanol-pyridine-water (6 : 4 : 3); Multi-development by Ascending;
▨ detected with aniline-phthalic acid
○ detected with anisidine-phosphoric acid
(a) was detected with above both reagents.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 281～286, 1960]

Transglucosidation Action of Crystalline Mold Maltase

Part II. Action of the Enzyme on Maltose and Isolation of Transglucosidation Products by Carbon Column Chromatography

By Shiro SUGAWARA, Yukihiko NAKAMURA and
Tokuji SHIMOMURA

Department of Agricultural Chemistry, University of Hokkaido, Sapporo

Received August 3, 1959

Crystalline maltase of "koji" mold or crystalline Takamaltase synthesized from maltose di-, tri-, tetra-, penta-, hexa- and heptasaccharides and also presumably octasaccharide. These saccharides were isomaltose, dextrantri-, tetra-, penta- and hexaose, and panose, 4-α-[dextrantri-, tetra-, penta-, hexa- and heptaosyl]-D-glucose. These saccharides were separated by chromatography on a carbon-celite column.

In the previous paper the authors reported on the glucosyl transfer from α-heterosides to alcohols and sugars by crystalline mold maltase and the enzyme was recognized as a transglucosidase[1].

Transglucosidases of mold have already been described by Pazur and French[2], Pan, Nicholson and Kolachov[3], and Tujisaka and Fukumoto[4]. These workers have investigated the action of transglucosidase on maltose principally and observed the formation of oligosaccharides from the disaccharide.

The authors accordingly examined the transglucosidation action of maltase on holo-glucoside, maltose, as a continuance of the previous experiment on heteroside. The results indicated that maltase of "koji" mold or Taka-maltase is identical with the transglucosidase described by the above investigators[2,3].

However in the present experiment, penta-, hexa- and heptasaccharides and presumably octasaccharide were found and isolated. These have hitherto not been observed in maltose-transglucosidase reaction.

The present paper deals with the qualitative and quantitative observation of maltase-maltose reaction, and the separation of products by carbon column chromatography.

EXPERIMENTAL

Methods and Material:

Crystalline maltase was isolated from "koji" mold and Takadiastase by the method reported in the previous papers[5,6]. The same behaviour of both enzymes toward transglucosidation action as well as substrate specificity had been observed in preliminary experiments.

Maltose was purified from a commercial source and no contamination of other sugars was examined. Isomaltose, panose, amylotri-, tetra and pentaose were kindly supplied by Prof. K. Aso. The sugars were determined reductometrically by a copper reagent of Somogyi.

In paperchromatography the solvent used was butanol-pyridine-water (6 : 4 : 3) and developed by a multiple ascending technique, elsewhere mentioned. The carbon used in column chromatography was a "Shirosagi-brand" Takeda commercial product.*

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[1] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **24**, 278 (1960).

[2] J. H. Pazur and D. French, *J. Biol. Chem.*, **196**, 265 (1952).

[3] S. C. Pan, L. W. Nicholson and P. Kolachov, *Arch. Biochem. Biophys.*, **42**, 421 (1953).

[4] Y. Tujisaka and J. Fukumoto, *Symp. on Enzyme Chem. Japan*, **10**, 84 (1958).

* Abbreviations:
G: glucose, M: maltose, D_2: isomaltose, D_3, D_4, D_5, D_6 and D_7: dextrantri-, tetra-, penta-, hexa-, and heptaose, D_2G: panose, D_3G, D_4G, D_5G, D_6G and D_7G:
4-α-[dextrantri-, tetra-, penta-, hexa- and heptaosyl]-D-glucose.

[5] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **23**, 156 (1959).

[6] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **24**, 112 (1960).

Paperchromatographical Observation of Transglucosidation Action of Crystalline Maltase on Maltose.

Preliminary experiments were performed by application of paperchromatographical analysis. The chromatogram showed that the enzyme acted on maltose to form several oligosaccharides as well as a hydrolysis product, glucose, and that the isomaltose formed remained throughout the incubation period, while panose almost disappeared after twenty-four hrs. The detection of oligosoccharides higher than D_4 was unsuccessful in this experiment, although these sugars were isolated by column chromatography as described in a later section (Fig. 1).

Quantitative analysis of the products was also made by paperchromatography and subsequent extraction of each sugar. Saccharides higher than panose were estimated in the lump, because of lack of distinctness in the detection of spots (Tab. I).

Separation of Transglucosidation Products in Maltase-Maltose Reaction by Carbon Column Chromatography.

The experiment was first performed using 5 g of maltose for the purpose of separation of reaction products and quantitative estimation. In the next experiment the authors attempted to prepare the products in a large scale and thus several saccharides which did not appear in the first experiment were found.

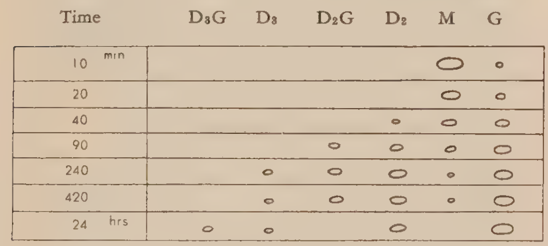

FIG. 1. Paperchromatogram of the Products formed by Maltose-maltase Reaction in Various Periods of Incubation

Maltose, 0.1 M; 0.5% enz. sol. 1 ml; acetate (pH 4.0) 3×10^{-2} M in 7 ml of reaction mixture. 45°C

Experiment 1.

Five g of maltose in 100 ml of 0.01 M acetate buffer (pH 4.0) was treated with 10 mg of crystalline maltase isolated from "koji" mold. The reaction mixture was allowed to stand at 35°C for twenty hrs. At the end of this period about 18% of maltose remained in the reaction mixture (Tab. II). The reaction products were adsorbed and chromatographed on a carbon-celite column (34~180 mm) (Fig. 2). Elution was first made with water to remove glucose, and isomaltose appeared in the successive water-effluents soon after the end of elution of glucose. Subsequently, the peak of isomaltose fraction was obtained in two parts, in both water and 5% alcoholic effluents. In the last fraction, eluted with 20% alcoholic solution, D_3G and D_5 were detected by paperchromatography. These two sugars were separated by paperchromatography and prepared by subsequent extraction, as will be described later.

Amounts of each saccharide formed were determined as glucose after the acid-hydrolysis of a portion of each fraction collected (Tab. II).

Isomaltose: The combined eluates (No. 7~41) were concentrated under diminished pressure and the syrup obtained was dissolved in hot methanol. The filtered methanol solution was evaporated to dryness in vacuo. As the residue was still syrupy, it was treated with ethanol. Isomaltose was obtained as white powder. Yield, 0.7 g. $[\alpha]_D=+117.7°$. Published value; 120°⁽⁷⁾, 119°⁽²⁾. Mobility in paperchromatography was identical with pure isomaltose.

TABLE I. QUANTITATIVE ANALYSIS OF THE PRODUCTS OBSERVED IN PAPERCHROMATOGRAM (FIG. 1)

Time of reaction	Glucose	Maltose	Isomaltose	Panose	Dextrantriose and others
0 min.		0.26 g	mg		mg
10	0.01	0.25			
40	0.02	0.27			
90	0.05	0.20	0.01	0.02	0.02
240	0.10	0.09	0.04	0.03	0.01
420	0.16		0.05	0.03	0.01
24 hrs	0.19		0.04	0.00	0.03

The sugars were extracted with 5 ml of boiling water from the corresponding spot of the chromatogram; spotted with 0.01 ml soln.; Conditions of the reaction were the same as those shown in Fig. 1

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[7] E. M. Montgomery, F. B. Weakley and G. B. Hilbert, *J. Am. Chem. Soc.*, **71**, 1682 (1949).

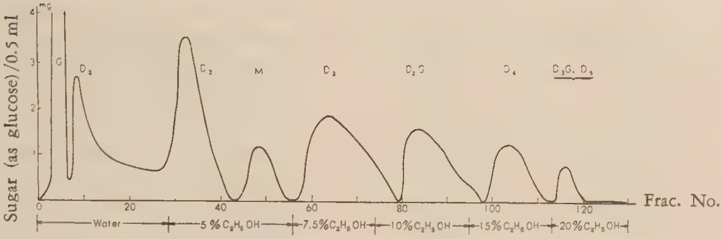

FIG. 2. Experiment 1. Chromatogram of Transglucosidation Products on Carbon-celite Column

Each 10 ml of the effluents, except each 50 ml of No. 1∼15, were collected. The sugars were determined reductometrically and calculated as glucose.

TABLE II. DETERMINATION OF AMOUNTS OF THE PRODUCTS OBTAINED BY THE MALTASE-MALTOSE REACTION

Glucose	1.79 g	35.8%
Isomaltose	1.09	21.8
Maltose, remaining,	0.91	18.2
Dextrantriose	0.46	9.2
Panose	0.46	9.2
Dextrantetraose	0.42	8.4
4-α-[dextrantriosyl]-D-glucose and dextranpentaose	0.12	2.4

Calculated as glucose after hydrolysis with 4 N-HCl, 6 hrs.

Dextrantriose: The combined eluates (No. 58∼77) were treated in the same manner as in the isomaltose fraction. After partial acid hydrolysis, G, D_2 and the remaining D_3 were detected by paperchromatography. $[\alpha]_D = +132°$, published value; +134°[2]. Yield; 310mg.

Panose: Panose was crystallized from methanol after undergoing the same treatment as fraction D_2. The R_F value was in accordnace with pure panose. $[\alpha]_D = +152°$, published value; +154°[8] +150°[2]. Yield; 300 mg.

Dextrantetraose: Small amounts of panose were contaminated in this fraction (No. 97∼110). The contaminant was removed by rechromatography on a carbon column. The isolation technique of D_4 was the same as described above. After partial acid-hydrolysis, G, D_2, D_3 and the remaining tetraose were identified by paperchromatography. $[\alpha]_D = +149°$. Yield; 250 mg.

4-α-[Dextrantriosyl]-D-glucose and Dextranpentaose: This fraction (No. 114∼118) was concentrated under diminished pressure. The syrup obtained was placed in a streak on filter papers (5×35 cm) and the resolution of the saccharides was accomplished by the multiple ascending technique. Then both sides of the resulting chromatogram were cut, and sprayed, and used as a mark to determine the corresponding bands of the two saccharides. The saccharides were extracted with boiling water from the paper. The combined extracts from eleven chromatograms were concentrated under diminished pressure and the saccharides were precipitated with ethanol and dried. Yield; D_3G, 40 mg and D_5, 50 mg. The acid-hydrolysis products were G, M, D_2, D_2G, D_8 and the remaining tetraose from the former, and G, D_2, D_3, D_4, and the remaining pentaose from the latter.

In this experiment D_5 was the highest polymer of glucose possessing only 1,6-α-glucosidic linkages, and mobilities of the dextran series of oligosaccharides on paper showed a linear relation, which had been experimentally found by French et al. (Fig. 3).

Maltase Action on Transglucosidation Products: By treating the transglucosidation products with maltase it was observed with paperchromatography that these saccharides function as cosubrtrates in the maltose-maltase reaction. For instance, D_3 was formed from D_2, and D_3 and D_3G from D_2G. Whereas with D_5 or D_3G not any substance except hydrolysis products was found. These reslts as well as the acid-hydrolysis products and specific rotation values are summerrized in Tab. III.

Experiment 2:

The purpose of this work was to prepare the transglucosidation products in a large scale. As the reaction of maltase has a tendency to proceed to hydrolysis when the ratio of the enzyme to the substrate is high, the ratio was extremly decreased.

Fifteen mg, of crystalline Taka-maltase and 115 g of maltose were dissolved in 1.5 l of water and buffered

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[8] S. C. Pan, L. W. Nicholson and P. Kolachv, *J. Am. Chem. Soc.*, **73**, 2547 (1951).

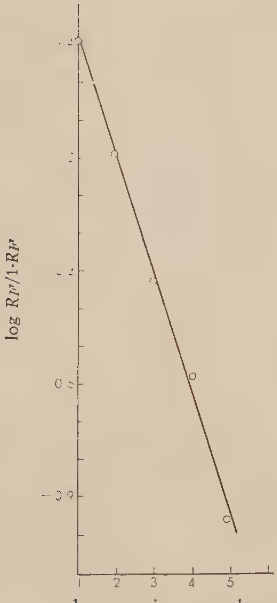

FIG. 3. Linear Relation of Mobilities of Dextran Series of Transglucosidation Products on Paper.

with 15 ml of 0.5 M-acetate (pH 5.0). The reaction mixture was allowed to stand for thirty hours at 30°C.

The reaction products were adsorbed and chromatographed on a charcoalcelite column (10 × 50 cm) in the same manner as described in Experiment 1. The effluents were collected in each 250 ml portion and a elution curve was made by measuring the reducing power (calculated as glucose) (Fig. 4).

The bulk of D_3G was separated from D_5 at a 17.5% concentration of ethanol (cf. Experiment 1).

The procedures of preparation of D_2, D_3, D_2G, D_4, D_3G and D_5 were the same as described in Experiment 1. Yield; D_2; 24 g, D_3: 8 g, D_2G: 21 g, D_4: 2.6 g, D_3G: 11.5 g, D_5: 2 g. The appropriate estimation of these sugars formed, calculated from reducing power, was as follows; G: 30.1%, M: 2.5%, D_2: 22.9%, D_3: 7.4%, D_2G: 17.4%, D_4: 2.3%, D_3G: 9.5%, D_5: 1.8%.

25% Ethanol Fraction (No.97~100): Three sugars were detected in this fraction by paperchromatography. Although the resolution and isolation were accomplished by the paperchromatographical technique described previously, the multiple descending technique was adopted.

One of saccharides obtained was D_3G contaminated. Yield; 65 mg. The two other saccharides were D_4G and D_6, and their tentative structures were suggested from the production of G, M, D_2, D_2G, D_3, D_3G and D_4 from D_4G, and C, D_2, D_3, D_4 and D_5 from D_6 by acid hydrolysis. Yield; D_4G, 220 mg; D_6, 100 mg. $[\alpha]_D$: D_4G, +212°; D_6, +178°.

30%~35% Ethanal Fraction (No. 103~107): This fraction consisted of a mixture of two saccharides, D_5G and D_6. In the paperchromatographical method for isolation of these sugars, the solvent was allowed to flow down for 48 hrs. The structure of D_5G was presumed from the production of G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G and D_5 by hydrolysis with acid. D_6 was identified from mobility on paper and its hydrolysis product from a comparison with D_6 isolated from the 25% ethanol fraction. Yield; D_5G, 80 mg; D_6, 60 mg.

40% Ethanol Fraction (No. 109~110) and 45% Ethanol Fraction (No. 112~113): The paperchromato-

TABLE III. PRODUCTS OBTAINED FROM BY PARTIAL ACID HYDROLYSIS OR MALTASE ACTION ON TRANSGLUCOSIDATION PRODUCTS, AND THEIR SPECIFIC ROTATION VALUE

	Products		
	Acid hydrolysis	Maltase action	
Isomaltose	G, (D_2)	G, (D_2), D_3	+117.7°
Dextrantriose	G, D_2, (D_3)	G, D_2, (D_3), D_4	+132°
Panose	G, D_2, M, (D_2G)	G, D_2, D_3, (D_2G), D_3G	+152°
Dextrantetraose	G, D_2, D_3, (D_4)	G, D_2, D_3, (D_4), D_5	+149°
4-α-[dextrantriosyl]-D-glucose	G, M, D_2, D_2G, D_3, (D_3G)	G, D_2, M, D_3, (D_3G)	+191°
Dextranpentaose	G, D_2, D_3, D_4, (D_5)	G, D_2, D_3, D_4, (D_5)	+158°

Acid hydrolysis: 0.1 N-H_2SO_4, 3 hrs, 100°C
Maltase action: 40 mg of substrate, 0.5 ml of 0.5% enz. sol., 0.5 ml of 0.1 M acetate (pH 4.0) in 5 ml
Brakets represent the remaining saccharides.

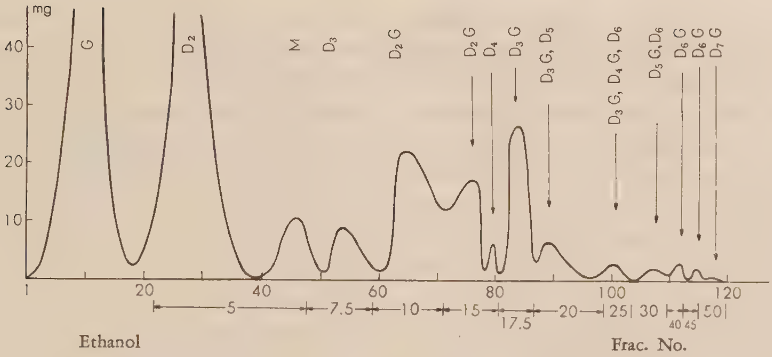

FIG. 4. Experiment 2. Chromatography of Transglucosidation Products on Carbon-celite Column.

gram indicated that only one saccharide is contained in both fractions and that mobilities are also the same each other. The saccharides were a heptasaccharide, D_6G, which produced G, M, D_2, D_3, D_4, D_5, D_6, D_2G, D_3G, D_4G and D_5G by acid hydrolysis. Yield; 370 mg (combined). $[\alpha]_D = +226°$.

50% Ethanol Fraction (No.114∼120): In this stage of elution, it was difficult to observe the appearance of the peak by measurement of reducing power. After No. 119 no saccharides were practically found. Accordingly, the collection of 50% alcoholic eluates was made by combining No. 114∼120 in the lump. The saccharide was prepared by concentration of combined eluates and precipitation with alcohol. Yield; 300 mg. G, M, D_2, D_3, D_4, D_5, D_6, D_2G, D_3G, D_4G, D_6G were found by partial acid hydrolysis. Mobility on paper was less than D_6G. From such results, the saccharide obtained from this fraction was presumed to be an octasaccharide. However, according to the reason described below, the presence of D_7 in this saccharide is most probable.

In paperchromatography, the transglucosidation products moved on paper in the following order; G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G, D_5, D_5G, D_6, D_6G, and D_7G. From this observation as well as the order of elution from the column, the probable appearance of D_7 was expected. However the mobility on paper was extremely low and was closely resemble to each other in such saccharides of higher molecular weight in the solvent system used, and subsequently by means of this method it was practically difficult to separate D_7 from D_7G.

DISCUSSION

In a previous paper in a study of the glucosyl transfer action of crystalline mold maltase from α-heteroglucoside to acceptors, it was reported that the enzyme is a transglucosidase. In the present study, this has been further confirmed by isolation and identification of many oligosaccharides produced by the action of this enzyme on α-hologlucoside, maltose.

The saccharides produced from maltose were di-, tri-, tetra-, penta-, hexa-, hepta- and octasaccharides: D_2, D_3, D_2G, D_4, D_3G, D_5, D_4G, D_6, D_5G, D_6G and D_7G. Among these eleven sugars, the saccharides possessing higher molecular weight such as D_4G, D_6, D_5G, D_6G and D_7G have been found and described for the first time.

Although a conclusive determination of the structure by preparing and analyzing the derivatives has not been accomplished, the experimental results present some evidences for the structure of these products formed from maltose. The mobilities of saccharides on paper were in accord with those of pure substances and specific rotation values were also practically identical with published values.

Most of the saccharides of higher molecular weight, which were found in this study, were analyzed by partial hydrolysis with acid and by subsequent paperchromatographical identification of the hydrolysis products. Naturally, in the partial acid-hydrolysis of such saccharides, some of the hydrolysis products obtained were the same as those characterized previously and tentatively in the same manner (Experiment

2). That is, most of the saccharides used as standards for the mobilities on paper were those that have been first isolated in the present study and yet had no conclusive structure. However the chromatographical technique was useful enough for characterizing such saccharides as transglucosidation products with mold maltase, since, from the results described hitherto by many investigators[2,4,9], it is natural to expect that a series of two types of sugar would appear during the reaction, corresponding to the difference of glucosyl acceptors-hydrolysis products, glucose, or the substrate, maltose itself.

An interesting phenomenon was found experimentally in paper- and column chromatography, and this observation also served as another basis for characterizing the saccharides. A series of sugar such as $D_2 \sim D_6$ which only contains 1, 6-linkages and another type such as $D_2G \sim D_6G$ which possesses a 1,4-linkage in the reducing terminal besides 1,6-linkage were eluted from the carbon-celite column and moved on paper alternately in the regular manner, as described below:

Order of elution: G, D_2, M, D_3, D_2G, D_4, D_3G, D_5, D_4G, D_6, D_5G

Order of mobility: G, M, D_2, D_2G, D_3, D_3G, D_4, D_4G, D_5, D_5G, D_6

In the two experiments on separation of the transglucosidation products, the amounts of each product were greatly differed. This may be explained from difference of reaction conditions, especially, the ratio of enzyme to substrate maltose. Since the reaction proceeds mildly when the ratio is low, the opportunities for liberated glucocse to act as a glcosyl acceptor would be less than those of substrate maltose itself. And the products may also function as cosubstrates. Accordingly in such case, the panose series of saccharides would accumulate in preference to the dextran series.

Octasaccharides were found to be the highest polymer among the transglucosidation products. But there remained the possibilty of contamination of dextranheptaose in this fraction as it was expected from the order of elution from the carbon column. At any rate, the yields of these saccharides of higher molecular weight were extremely low, as compared with the yields of di-, tri-, tetra- and pentasac- charides. Whether such degree of polymerization as octasaccharide is a limiting extent of polymerization or not, is a problem left for further investigation hereafter.

SUMMARY

Isomaltose, dextrantri-, tetra-, penta- and hexasaccharides, panose, and 4-α-[dextrantri-, tetra-, penta-, hexa- and heptaosyl]-D-glucose were isolated as transglucosidation products formed in the reaction of crystalline mold maltases with maltose.

Acknowledgement The authors are indebted to Professor K. Aso of Tohoku Univ., for kindly supplying the pure saccharides, and also wish to thank the Sankyo. Co. Ltd., for kindly supplying the Takadiastase used in this work.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 287~295, 1960]

Studies on Dry Yeast (VI)

Change of Dry Yeast in the Fermenting Activity and the MB-stainability during Storage at 0° and 30°C

By Masayoshi TAKAKUWA

College of Agriculture, Ehime University

Received May 25, 1959

When the sample was taken from the fermenting dry bakers' yeast suspension, the ratio of yeast cells negative to methylene blue staining (MBN) was calculated easily and exactly. MBN decreased more slowly than any of three factors of fermenting power, which were shown with three quotients (Q_{co_2} (20′~40′), ΔQ_{co_2}, Q_{co_2} (max)), during the storage of the dry yeast. ΔQ_{co_2} was the most inconstant of three quotients and the inconstancy was particularly clear in storage at 0°C, where others were almost constant.

When bakers' yeasts are dried to a certain moisture content, the rate of the decrease in the fermenting activity varies with the fresh yeast used as the material: the activity in one material decreases to 60% but in another material to 40%, even if their activities were almost the same in the fresh condition[1~3]. Such a relation is recognized also when dry yeasts are stored[2,3]. In the dry yeasts which have the same fermenting activity soon after dehydration, one loses much fermenting activity in only a few days but another does not lose so much even after a few months. The essential and sufficient qualities which the bakers' yeast must have in order to be made into the dry yeast have not yet been found. The content of cell components, e.g., total carbohydrate and total nitrogen, has been hitherto taken as a criterion in preparing the dry yeast[4~6]. But how such components protect the fermenting power from its decrease has been studied very little[7]. As these components were found to change very slightly in the author's experiment[8], the yeast cells which have the optimum content of these components might be given a physiological condition suitable to the dry yeast.

But, before looking into such a physiological condition, it might be necessary to examine again whether the fermenting power of the dry bakers' yeast may be taken not as a matter of free enzymes such as "zymase", but as that of yeast cells. Whether or not the fermenting power of dry yeast depends upon the living cells was formerly a matter of debate[9,10]. At present, the power is regarded as that of the living cell[11], but the relation between the fermenting power and the ratio of the number of living cells to total cells (the ratio of living cells) has not yet been studied in detail. Fink devised the methylene blue staining procedure to distinguish dead cells from living cells[12] and this procedure has been utilized as a simplified

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method to determine the ratio of living cells[1]. So, in this report, the ratio of yeast cells negative to methylene blue staining (MBN) which was determined according to the Fink's method was taken as a value to suggest the ratio of living cells. The relation between MBN and the fermenting power was looked into statistically, and it was confirmed that a high positive correlation exists between these values. Consequently, we should be aware of the most suitable physiological conditions of cells for preparing the dry bakers' yeast.

For ascertaining such physiological conditions, it might be useful to examine how yeast cells change generally in the process of dehydration or storage after dehydration. Formerly, the author found that the fermenting power of the dry bakers' yeast can be represented by three factors[13]: the initial fermenting activity; the power which recovers fermenting activity; the maximum fermenting activity to be recovered. Therefore, in this report, the retentions of MBN and these three activities were examined during the storage of dry yeast, and the relations between them were also examined.

MATERIALS AND METHODS

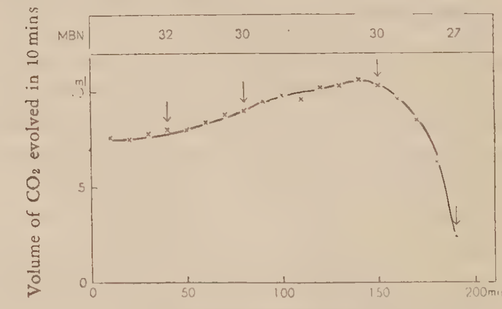
The preparation and the storage of dry yeast were carried out in the same way as in the author's previous report[2], i.e., air-dried at 30°~35°C and stored at 30°C, except the samples described particularly. The determination of the fermenting power was carried out also according to the same report and the fermenting power was shown with three quotients[13]: Q_{CO_2} (20′~45′) (the initial fermenting activity); ΔQ_{CO_2} (the power which recovers fermenting activity); Q_{CO_2} (max) (the maximum fermenting activity to be recovered). The moisture content was measured through drying 1 g of dry yeast at 100°~105°C till its constant weight was attained. The measurement of the number of living cells was carried out according to the Fink's method, where the cell which is not stained with methylene blue (0.01%) in the phosphate buffer of pH 4.6 is regarded as a living cell. But the yeast cells used in this measurement were sampled from the suspension after the determination of the fermenting activity. The suspension had been shaken as usual till the evolution of the maximum volume of carbon dioxide, or for exactly eighty minutes. Besides, in this report, the yeast cells which were not stained with methylene blue and had a clear outline of the cell membrane were regarded to be MB-negative, and the ratio of these MB-negative cells to the total cells (usually 500 cells) was shown with percentage. This value is represented by MBN. The error in the measurement was 1.0~2.7% (Table I) and little change was seen till the evolution of the maximum carbon dioxide as shown in Fig. 1. K. Shinma et al. found that the measurement in the Fink's method should be carried out within three minutes after mixing yeast suspension and methylene blue solution[14], but, in this method, no dif-

TABLE I. CRITERION ON STAINING WITH METHYLENE BLUE

Name of Test	Number of Sampling	Not Stained %	average	error*
1-A	1	33	34	1.0
	2	34		
	3	35		
1-B	1	32	34	1.6
	2	35		
	3	34		
2	1	49	48	1.2
	2	47		
	3	47		
3	1	33	35	2.7
	2	33		
	3	37		
	4	38		
	5	32		

* Error is shown by standard deviation.

Note: The sample in 3 is a dry baker's yeast stored for 500 days at 0~5°C. 1-A, 1-B were taken from the same sample which were shaken in different vessels.



Time of Fermentation

FIG. 1. Relation Between MBN and Fermenting Activity in Process of Fermentation

↓ shows the time when sample was taken and MBN measured.

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13) M. Takakuwa, This Bulletin, **23**, 316 (1959).

14) K. Shinma, T. Kada and M. Ikeda, unpublished data

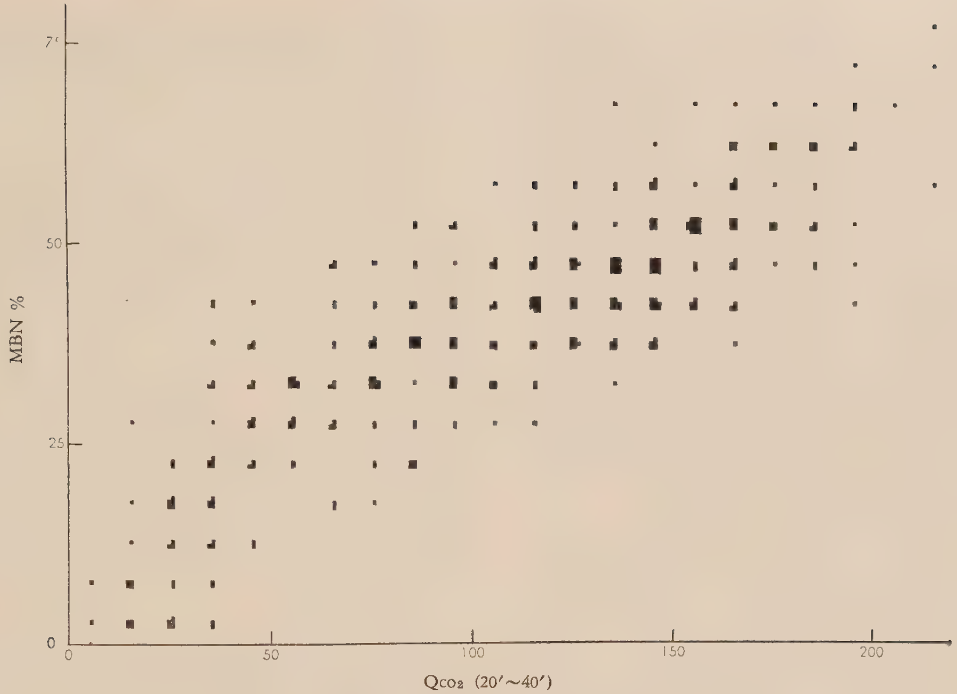

FIG. 2. Correlation Between MBN and Q_{CO_2} (20′~40′)

A size of ■ shows frequency.

ference was found between the value measured within five minutes and that within sixty minutes.

RESULTS

1) The relation between MBN and Q_{CO_2} (20′~40′) The relation was studied with 390 examples, which consisted of all values measured soon after dehydration and during storage not only in the air-dried yeast but also in the dry yeast prepared with various methods[3]. The distribution is shown in Fig. 2. It was deduced that the positive correlation ($r=0.839$) exists between them. But each variance of MBN or Q_{CO_2} (20′~40′) in a certain Q_{CO_2} (20′~40′) or MBN was of considerable extent as seen on this figure.

2) The relation between the moisture content and the retention of MBN during storage at the various temperature. As shown in Fig. 3, the retention of MBN during storage at various temperatures (0°~5°C, 20°C, 30°C, 37°C) was examined with the dry yeasts of various moisture contents. In this case, the state of the retention was observed

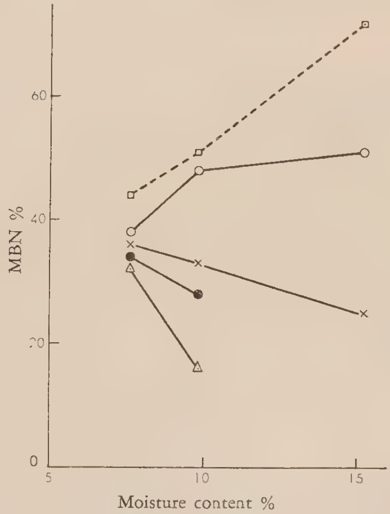

FIG. 3. Relation Between Moisture Content and MBN

□- - -□ soon after drying ; ——after 30 days of storage in various temperatures ; ○——○ 0°~5°C, ×——× 15°~20°C, ●——● 30°C, △——△ 37°C No cell of 15.2% sample was not stained after 30 days in 30°C or 37°C.

with each of the samples which were air-dried. There was seen a tendency similar to the retention of fermenting activity, which was reported previously[2]. That is to say, the lesser the moisture content of the dry yeast, the smaller was the value of MBN when measured soon after drying, but, the lesser the content the larger was the retention of MBN after the storage of the dry yeast. The relation between the moisture content and MBN after the storage was opposite to that soon after dehydration; i.e., the lesser the moisture content of a sample, the lesser was the influence of the storage temperature. In other words, the sample of the less moisture content retained MBN during the storage at the higher temperature.

The retention ratios of Qco_2 (20′~40′) and MBN during storage were almost the same at 0°~5°C as shown in Fig. 4, but, when the temperature became high, the ratio of Qco_2 (20′~40′) decreased sooner than that of MBN. Even at the low temperature, the moisture content had influence on both ratios after the long period of storage. Both ratios were almost the same in the sample of 7.6% of the moisture content, but became very different in the sample of 10.0%. Hereafter the retention ratios are abbreviated to RMBN and $RQco_2$ (20′~40′), respectively.

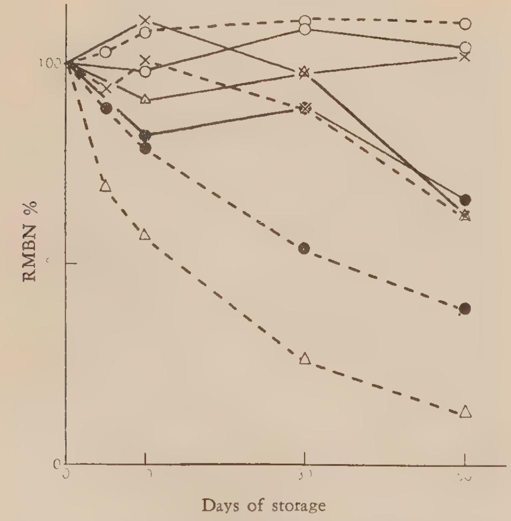

FIG. 4. Retention Ratios of MBN and Qco_2 (20′~40′) in Storage at Various Temperatures

—— RMBN, ---- $RQco_2$ (20′~40′)
○ 0°~5°C, × 20°C, ● 30°C, △ 37°C

3) As the fermentation of dry yeast depends not only upon Qco_2 (20′~40′) but also upon ΔQco_2 and Qco_2 (max), their retention ratios were calculated also and are shown as $R\Delta Qco_2$ and $RQco_2$ (max), respectively. Which factor has more connection with MBN during the storage at 30°C became the next subject for study.

(i) There was a positive correlation between $RQco_2$ (20′~40′) and RMBN ($r=0.741$): Each RMBN was usually larger than its corresponding $RQco_2$ (20′~40′) as shown in Fig. 5. This might support certainly the above findings that Qco_2 (20′~40′) decreases sooner than MBN during storage.

(ii) There was a very high positive correlation between $RQco_2$ (20′~40′) and $RQco_2$ (max) ($r=0.970$): Like RMBN $RQco_2$ (max) was larger than the corresponding $RQco_2$ (20′~40′) as shown in Fig. 6. This might suggest that Qco_2 (max) does not decrease sooner than Qco_2 (20′~40′) in the storage at 30°C. Moreover, when the values of A and B were calculated from the following equation: $A = RMBN - RQco_2$ (max), $B = RMBN - RQco_2$ (20′~40′), the relation of $A > B$ existed in 3 out of 23 examples, $A = B$ in 4, and $A < B$ in 16. So MBN might be deduced to have more connection with Qco_2 (max) than with Qco_2 (20′~40′).

(iii) r between RMBN and $R\Delta Qco_2$ was -0.039 in 43 examples. So the existence of the correlation between them was not regarded as of any significance.

Therefore, considering the results of (i), (ii) and (iii), MBN might have more connection with Qco_2 (max) than Qco_2 (20′~40′) or ΔQco_2, in three quotients.

4) The positive correlation existed between $RQco_2$ (20′~40′) and $R\Delta Qco_2$ as shown in Fig. 7 ($r=0.611$). The examples on this figure were limited to those of $R\Delta Qco_2$ smaller than 180, but the

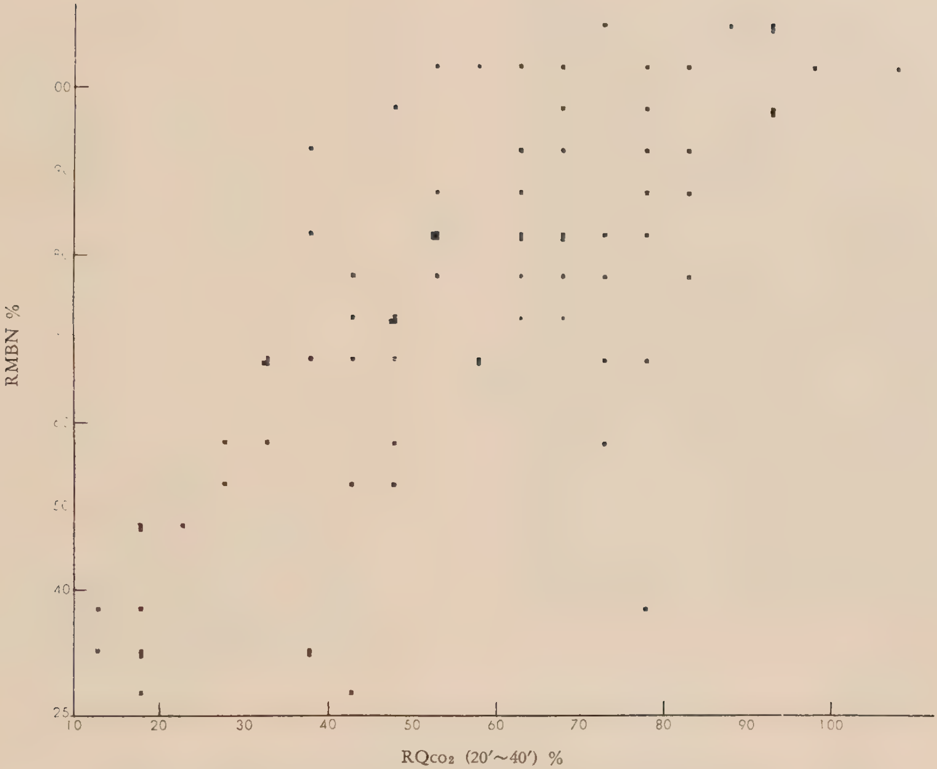

FIG. 5. Correlation between Retention Ratios of MBN and Q_{CO_2} (20′∼40′)

ΔQ_{CO_2} was sometimes so small (0 or near to 0) that some $R\Delta Q_{CO_2}$ were ∞ or very large. Zero of ΔQ_{CO_2} measured soon after drying was seen in the dry yeasts prepared from 8 out of 28 materials of the fresh yeast, where Q_{CO_2} (max) was equal to Q_{CO_2} (20′∼40′). This might mean that the value of Q_{CO_2} (max) is often near to that of Q_{CO_2} (20′∼40′) when they are measured soon after drying. As shown in the figure, moreover, when Q_{CO_2} (20′∼40′) decreased much, the degree of the decrease was larger in ΔQ_{CO_2} than in Q_{CO_2} (20′∼40′), while, when Q_{CO_2} (20′∼40′) decreased only a little as in an initial period of the storage, ΔQ_{CO_2} decreased less than Q_{CO_2} (20′∼40′), or ΔQ_{CO_2} increased. It might be deduced from these results that the initial fermenting activity decreases in the initial period of storage and the induction period appears and then the decrease of ΔQ_{CO_2} (the increase of the induction period) goes on more rapidly than that of Q_{CO_2} (20′∼40′).

5) **The dry yeast of a moderate moisture content retains an almost constant Q_{CO_2} (20′∼40′) even after about one and a half years in the storage at 0°C:** MBN also was retained almost constant during the storage at the low temperature. When a sample which had been stored previously at 0°C was stored at 30°C, however, both Q_{CO_2} (20′∼40′) and MBN decreased more rapidly than those of the sample which was stored at 30°C soon after drying (Fig. 8). This fact might suggest that some change which is not recognized from values of Q_{CO_2} (20′∼40′) and MBN had occurred in the storage at 0°C. So RQ_{CO_2} (20′∼40′) and RQ_{CO_2} (max) in the storage at 0°C were compared with each other and it was

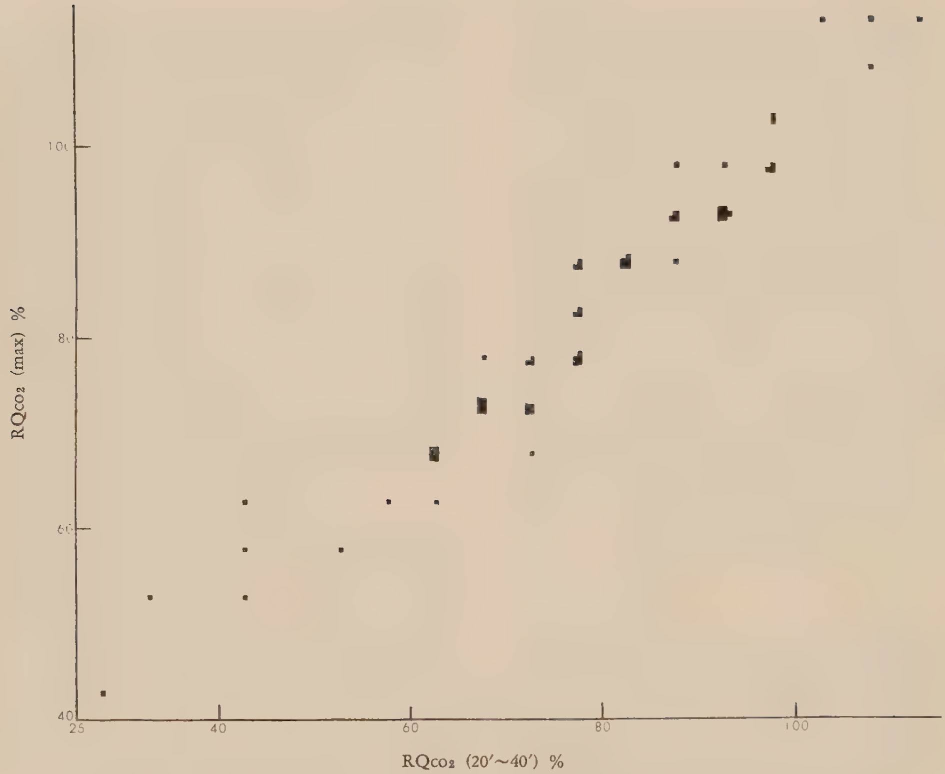

FIG. 6. Correlation between Retention Ratios of Qco_2 (20′∼40′) and Qco_2 (max)

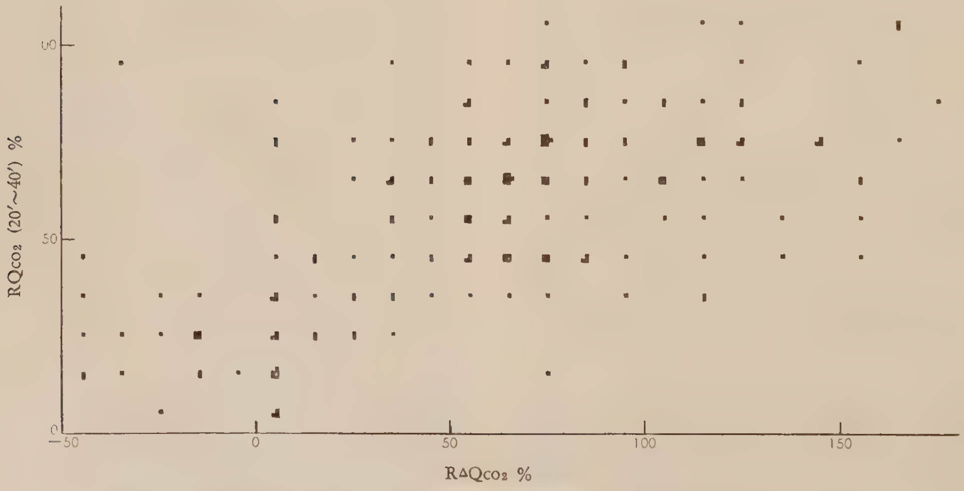

FIG. 7. Correlation between Retention Ratios of Qco_2 (20′∼40′) and ΔQco_2

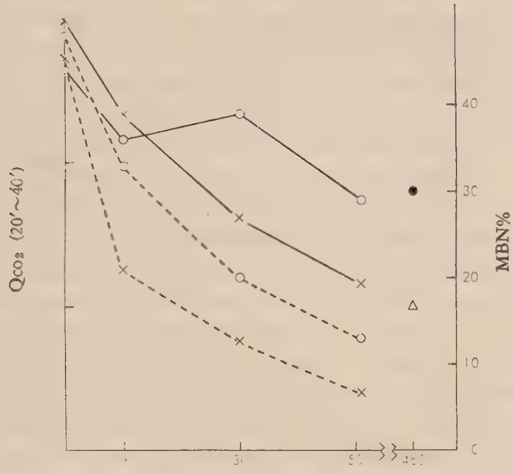

FIG. 8. Effect of Previous Storage at 0°C to Storage at 30°C

—— soon after drying, ---- stored previously at 0°C for 460 days. ○ MBN, × Qco$_2$ (20'~40'). ● and △ show MBN and Qco$_2$ (20'~40') respectively in the sample stored for 410 days at 0°C after 50 days of storage at 30°C.

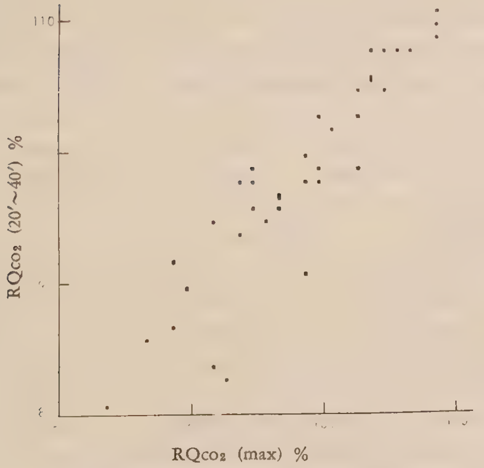

FIG. 9. Correlation between Retention Ratios of Qco$_2$ (20'~40') and Qco$_2$ (max)

found that Qco$_2$ (20'~40') did not decrease more rapidly than Qco$_2$ (max) in the retention ratio over 95% (Fig. 9); unlike the fact in the storage at 30°C. Also in the sample after the storage at 0°C, the relation between RMBN and RQco$_2$ (20'~40') was RMBN>RQco$_2$ (20'~40') during the storage at 30°C like the one stored at 30°C soon after drying (Fig. 8). The retentions of ΔQco$_2$ and Qco$_2$ (20'~40') in the storage at various temperatures varied especially in the low temperature, as shown in Fig. 10. That is to say, at 0°~5°C, Qco$_2$ (20'~40') was almost constant but ΔQco$_2$ remarkably inconstant. Besides, when the sample stored for four hundred days at 0°C after fifty days of the storage at 30°C was compared with the sample stored for fifty days at 30°C after four hundred days at 0°C, the former was much larger than the latter in both MBN and Qco$_2$ (20'~40') (Fig. 8). The sum of the above results is that, in storage at a low temperature (0°~5°C), the fermenting activity and MBN hardly changed but became labile to increase of temperature, and that, at 0°C, only ΔQco$_2$ was the most inconstant in three quotients of fermenting power of dry yeast.

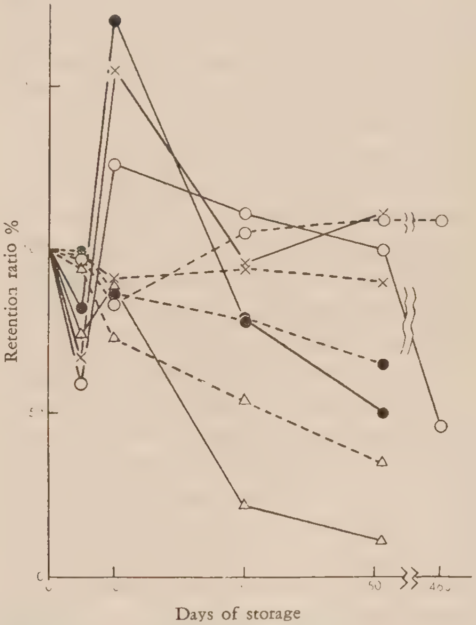

FIG. 10. Retention Ratios of ΔQco$_2$ and Qco$_2$ (20'~40') in Storage of Various Temperatures

—— R ΔQco$_2$, ---- RQco$_2$ (20'~40')
○ 0°~5°C, × 20°C, ● 30°C, △ 37°C

DISCUSSION

Fink counted the number of cells stained deep blue with methylene blue as that of dead cells to calculate the ratio of living cells[12)], but Shinma et al. modified this method and counted the number of cells not stained instead of the ones stained deep blue[14)]. Shinma et al. also found that the ratio of unstained cells varies with various conditions and decided definitely the time for suspending cells with shaking and other conditions.

But it is affirmed in this paper that, when the sample was taken from the yeast suspension after measuring the fermenting activity, the difference between stained cells and unstained ones was clearer and the method did not need such strict conditions as the time of measurement. It may be convenient also for the sufficient dispersion of cells to take the sample from the yeast suspension which was fermented in a shaking apparatus. It is difficult to distinguish the unstained cells from the partly stained cells or the lightly-stained cells. Perhaps such weakened cells as the latter die out and other quite living cells strengthen the living force, when the cells are allowed free action in controlled situations, the difference between the stained and the unstained ones becomes clear. Strengthening the living force is supposed easily from the fact that the fermenting activity of dry yeast is usually recovered in the process of fermentation. Besides, the fact that little increase in the ratio of unstained cells was recognized in the process of fermentation suggests probably that ΔQ_{co_2} does not depend upon the increase of the number of the yeast cells.

The fact that out of the three quotients of fermenting power during the storage at 30°C RMBN had the most connection with RQ_{co_2} (max) may suggest that the value of each Q_{co_2} (max) can be estimated from the value of each MBN in comparing several samples, even if the measurement is not continued for several hours till the appearance of Q_{co_2} (max). The fact that the relation between MBN and the moisture content was similar to that in Q_{co_2} $(20'\sim40')$ during the storage at various temperatures might suggest that even a little difference in moisture content is closely connected with the life of cells even after the cells have been dried till within the bound water.

As the fermentation of the dry yeast depends upon the living cells, it may be supposed naturally that the fermentation of dry yeast is controlled by more varied factors than that of zymase. On the other hand, the fermentation of dry yeast is also different from that of fresh yeast. Besides, the state of the former varies according to the method of preparing or storing dry yeast. These facts might show that some change is made in the enzyme systems of yeast cells when yeast cells suffer from any damage, and that the change of function of the enzymes varies with the state of the damage of the yeast cells.

As the Q_{co_2} $(20'\sim40')$ is near to the Q_{co_2} (max) generally, ΔQ_{co_2} increases apparently in the initial period of storage. But perhaps ΔQ_{co_2} does not increase in reality. It looks probably so because Q_{co_2} $(20'\sim40')$ is very near Q_{co_2} before storage. And since the dry yeast cell soon after dehydration holds a very strong power in recovering the fermenting activity, such a cell shows almost the same value in Q_{co_2} $(20'\sim40')$ as in Q_{co_2} (max), which shows that this cell is very similar to a fresh cell. Therefore the appearance of the induction period might depend on the decrease of ΔQ_{co_2} as well as that of the initial fermenting activity.

When the cell stored previously at 0°C was stored at 30°C, the decrease of MBN and Q_{co_2} $(20'\sim40')$ was more rapid than when the cell was stored at 30°C without the previous storage at 0°C. This might mean that the change in the storage at 0°C made the cell labile to the increase of the storage temperature.

SUMMARY

When the sample in the Fink's method of staining yeast cells with methylene blue was taken from the yeast suspension after deter-

mining the fermenting activity, the measurement was carried out more easily and exactly. Next the existence of the positive correlation ($r=0.839$) was confirmed between the ratio of yeast cells negative to methylene blue staining (MBN) and Q_{CO_2} (20′∼40′). So the change of dry yeast during storage was examined concerning MBN and three quotients of fermenting power (Q_{CO_2} (20′∼40′), ΔQ_{CO_2}, Q_{CO_2} (max)). Besides, the interrelation of these quotients was discussed in connection with the influence of the storage temperature.

1) The relation between moisture content and MBN retention of dry yeast in the storage at various temperatures (0°∼5°, 20°, 30°, 37°C) was similar to that reported formerly about Q_{CO_2} (20′∼40′). The lesser the moisture content of dry yeast, the smaller was the value of MBN but the value retained during the period of storage was larger. Besides, the lesser the moisture content, the lesser was the influence of the storage temperature. But such a tendency in the storage was seen more clearly in Q_{CO_2} (20′∼40′) than in MBN.

2) In the storage at 30°C, the relation between the retention ratios of MBN and above three quotients was examined. In the initial period of storage, Q_{CO_2} (20′∼40′) decreased and the induction period appeared. Afterwards, the decrease of ΔQ_{CO_2} (the increase of the induction period) went on more rapidly than that of Q_{CO_2} (20′∼40′). Of the three quotients Q_{CO_2} (max) decreased most slowly. MBN decreased more slowly than Q_{CO_2} (max), and had more connection with Q_{CO_2} (max) than with Q_{CO_2} (20′∼40′) or ΔQ_{CO_2}, in the three quotients.

3) The decrease of MBN was slowest also in the storage at 0°C. Q_{CO_2} (20′∼40′) also was almost constant in the case of dry yeast of a moderate moisture content, but only ΔQ_{CO_2} was clearly inconstant.

4) In the samples stored at 30°C after the storage at 0°C, the decrease of MBN and Q_{CO_2} (20′∼40′) was more remarkable than in those stored at 30°C soon after drying. On the contrary, both MBN and Q_{CO_2} (20′∼40′) decreased only a little when the same samples were stored at 0°C after the storage at 30°C.

Acknowledgment. The author wishes to express his sincere thanks to Prof. I. Yamasaki of Kyushu University for his guidance in this work.

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Studies on the Fermentative Preparation of L-Aspartic Acid from Fumaric Acid*

By Masahiko KISUMI, Yoshitaro ASHIKAGA and Ichiro CHIBATA

*Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd.***

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Among a number of organisms *Pseudomonas fluorescens* 6009-2 and mutant Ki-1023 derived from *Escherichia coli* K-12 by ultraviolet radiation were chosen as suitable strains for the accumulation of L-aspartic acid from fumarate and ammonium salts.

By employing these organisms, optimum cultural conditions for the production of L-aspartic acid were investigated. As a result, L-aspartic acid was obtained in a good yield by the advantageous process presented below.

In recent years, a number of reports concerning amino acid fermentation with glucose as the carbon source have appeared. Whereas, on the other hand, the authors have been studying the biosynthesis of amino acids employing fumarate as the main carbon source, for fumaric acid is readily available and is considered to be suitable as a starting material for amino acid production.

In this paper the results of studies on the fermentative production of L-aspartic acid, which plays an important rôle in amino acid metabolism are presented.

Since Quastel and Woolf[1] have observed the reversible fomation of aspartate from fumarate and ammonium ion in the presence of cell suspension of *Escherichia coli*, many reports have been published on the aspartase which catalyzes the above reaction. Although a practical preparation of L-aspartic acid has only been shown in reports by the above mentioned Quastel and by Sumiki[2] using resting cells of yeast, no report on the production of L-aspartic acid by a fermentative procedure has been published.

In the course of preparing this manuscript Kitahara[3] and Kinoshita[4] have reported on the formation of L-aspartic acid by the addition of fumarate to the culture after completion of growth of the organism, and Kinoshita has disclosed that the addition of fumarte from the initials tate of cultivation resulted in an unfavorable formation of aspartate.

The authors have screened a number of organisms to test the ability of formation and accumulation of aspartate from fumarate, and *Pseudomonas fluorescens* 6009-2 and a mutant Ki-1023 of *Escherichia coli* K-12 was chosen as the most suitable organism for the production of L-aspartic acid. With these organisms various conditions of fermentation have been studied. As a result, the conversion of fumarate to aspartate attained was almost agreed to the theoritical and pure L-aspartic acid was obtained in a yield over 85%.

MATERIALS AND METHODS

Orgamisms Twenty strains of bacteria, 5 strains of streptomyces, 20 strains of molds and 15 strains of yeasts were used for the screening test. These organisms were supplied from the Institute of Applied Microbiology of Tokyo University, the Department of Fermentation Technology of Osaka University, and the Research Institute for Microbiological Diseases of Osaka University. Besides these organisms, 60 mutants of *Escherichia colk*-12 which were isolated by the authors with UV radiation and subsequent Penicillin screening, were also employed for the experiments.

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* Presented at the Symposium of Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 10, 1959.

** 962 Kashima-cho, Higashi-yodogawa-ku, Osaka.

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[2] Y. Sumiki, *Bull. Jap. Soc. Ferment.*, **23**, 33 (1928).

[3] K. Kitahara, S. Fukui and M. Misawa, Presented at the meeting of Kanto Division of Agricultural Chemical Society of Japan, Tokyo, Nov. 22, 1958.

[4] S. Kinoshita, K. Nakayama and S. Kitada, *Hakko Kyokaishi*, **16**, 517 (1958).

Composition of the Media The culture media employed for the experiments are shown in Table I.

Unless otherwise noted, the pH of medium is adjusted to pH 7.0.

Screening Experiments Slant cultures of tested organisms were carried out on the medium containing 1% peptone, meat extract and yeast extract for 1～3 days in the case of bacteria and streptomyces, and on malt extract medium (10° Bg.) for 2～4 days in the case of molds and yeasts at respective optimum temperature for growth.

The respective media (S-1～S-4) were distributed in amounts of 3 ml to test tubes and then sterilized. After inoculation with tested organisms of the slant cultures, stationary and shaking cultures were carried out for 2～10 days at 30°C or 37°C.

Routine identification and rough quantitative estimation of the formed amino acids was made by paper chromatography of the cultural broth. Paper chromatography was carried out with the ascending method using Tôyô filter paper No 51 and butanol : acetic acid : water = 4 : 1 : 1 and phenol : water = 4 : 1 as solvents. After development the chromatograms were sprayed with 0.2% ninhydrin in water-saturated butanol, and color development of the spots was measured.

Fermentation Experiments Fermentation experiments with selected organisms were carried out using media A-1～A-32 in Table I.

The respective media were distributed in amounts of 150 ml to 500 ml shaking flasks and were sterilized by autoclaving. After inoculation with two loopfuls of inoculum, the cultures were incubated for the desired length of time at 30°C or 37°C with reciprocal shaking (110 c.p.m., stroke 8 cm) or without shaking.

Analysis of the cultured broth was carried out as described in the following paragraph.

Methods of Analysis The assay of L-aspartic acid of the supernatant solution of the culture broth was carried out by ordinary microbioassay using *Leuconostoc mesenteroides* P-60. Besides microbioassay, paper chromatography was simultaneously carried out in order to detect the byproducts such as glutamic acid⁵⁾ and asparagine⁶⁾ which were found to influence the microbioassay of aspartic acid.

Fumaric acid was determined spectrophotometrically⁷⁾. Namely, a properly diluted supernatant solution of culture broth was measured by absorption at 250 mμ with a Beckman-type spectrophotometer.

RESULTS AND DISCUSSION

Screening Experiments of Organisms

As a result of the screening experiments carried out by the method previously described, the accumulation of aspartic acid was found in almost all of the tested bacteria, while, no or only a scarce accumulation was observed in the case of mold, streptomyces and yeasts under the conditions employed by the authors.

Of the tested bacteria, *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Bacillus megatherium* and *Bacillus subtilis* showed higher activity of aspartic acid formation from fumarate. Formation of other amino acids such as glutatmic acid and alanine, which were most commonly found in these experiments, was less in the case of *Escherichia coli* and *Pseudomonas fluorescens* than in the other strains.

From the above findings, *Escherichia coli* K-12 Ki-1023 (methionine requiring mutant) and *Pseudomonas fluorescens* 6009-2 were selected as the most favorable strains for the author's purpose.

On the Cultural Conditions for L-Aspartate Accumulation from Fumarate

In order to establish the most advantageous cultural conditions for the production of L-aspartic acid from fumaric acid, various conditions were investigated with *Escherichia coli* K-12 Ki-1023 and *Pseudomonas fluorescens* 6009-2 which were chosen by the above screening experiments.

I) Effect of Ammonium Salts

It is quite possible that the formation of aspartate is influenced by the kind of ammonium

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5) G. Tamura, T. Sumita, G. Kirimura and S. Miyazawa, *J. Agr. Chem. Soc. Japan*, **26**, 464 (1952).

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TABLE I. COMPOSITION OF THE MEDIA

Medium	Glucose (%)	Fum-Na (%)	Fum-NH_4 (%)	Inorg. NH_4 Source (%)		Org. N Source (%)		$CaCO_3$ (%)
S–1	2.0	2.0	–	$(NH_4)_2SO_4$	1.0	Yeast ext.*	0.2	–
S–2	1.0	3.0	–	//	2.0	Peptone Yeast ext.	0.2 0.5	–
S–3	–	3.0	–	//	1.0	Yeast ext.	0.5	–
S–4	0.5	5.0	2.0	–		Peptone Yeast ext.	0.2 1.5	1.0
A–1	//	3.4	3.2	–	–	Peptone Yeast ext.	0.2 0.5	–
A–2	//	6.9	–	NH_4Cl	2.3	//		–
A–3	//	//	–	$(NH_4)_2HPO_4$	2.8	//		–
A–4	//	//	–	NH_4NO_3	3.5	//		–
A–5	//	//	–	$(NH_4)_2SO_4$	2.8	//		–
A–6	//	//	–	$(NH_2)_2CO$	1.3	//		–
A–7	//	4.3	2.4	–		//		–
A–8	//	2.5	4.0	–		//		–
A–9	//	1.8	4.8	–		//		–
A–10	//	0.9	5.7	–		//		–
A–11	//	–	6.5	–		//		–
A–12	//	3.4	3.2	–		//		1.0
A–13	–	//	//	–		//		2.0
A–14	0.5	//	//	–		//		//
A–15	2.0	//	//	–		//		//
A–16	5.0	//	//	–		//		//
A–17	10.0	//	//	–		//		//
A–18	0.5	1.4	1.3	–		//		1.0
A–19	//	6.9	6.5	–		//		//
A–20	//	13.8	13.0	–		//		//
A–21	//	3.4	3.2	–		Casein hyd.	1.1	//
A–22	//	//	//	–		Corn steep liq.	2.2	//
A–23	//	//	//	–		Peptone	0.9	//
A–24	//	//	//	–		Yeast ext.	6.2	//
A–25	//	//	//	–		Meat ext.	7.1	//
A–26	//	//	//	–		Peptone Meat ext.	0.9 0.9	//
A–27	//	//	//	–		Peptone Yeast ext.	0.7 1.8	//
A–28	–	//	//	–		Peptone	0.9	–
A–29	–	//	//	–		//		1.0
A–30	–	–	6.5	–		//		–
A–31	–	–	6.5	–		//		1.0
A–32	–	6.9	–	NH_4Cl	2.3	//		//

All media contain 0.2% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$.

* Figure indicates percentage as dried yeast.

TABLE II. EFFECT OF AMMONIUM SALTS

Medium	Ammonium salt	Strain*	Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
			Days of culture							
			2	4	6	9	2	4	6	9
A-1	Fum.-NH_4	P	18	45	46	47	3	26	28	30(55)**
		E	7	15	35	40	2	8	12	13(28)
A-2	NH_4Cl	P	7	8	14	19	1	2	1	2
		E	4	11	33	45	1	6	12	35(68)
A-3	$(NH_4)_2HPO_4$	P	8	28	36	40	1	2	1	2
		E	13	43	44	43	1	11	13	16(33)
A-4	NH_4NO_3	P	5	6	10	22	2	1	2	2
		E	5	7	11	13	2	2	3	3
A-5	$(NH_4)_2SO_4$	P	12	34	42	46	0	2	2	2
		E	11	11	29	47	1	3	5	2
A-6	$(NH_2)_2CO$	P	0	0	1	0	0	0	0	0
		E	0	0	1	4	0	0	0	1

Shaking culture was carried out at 30°C.
* P and E represent *Pseudomonas fluorescens* 6009-2 and *Escherichia coli* K-12 Ki-1023, respectively.
** Figure in round brackets represents conversion ratio from consumed fumarate.

TABLE III. EFFECT OF MOLAR RATIO OF FUMARATE AND AMMONIA

Medium	Ratio	Strain	Fum. cons. (mg/ml)					Asp. form. (mg/ml)				
			Days of culture									
			2	4	6	9	12	2	4	6	9	12
A-7	1:0.75	P	18	40	44	45		4	14	29(57)	19:25*	
		E	8	16	35	44		2	5	8	11(21)	
A 1	1:1	P	16	45	46	46	48	3	35(68)	35	31	29
		E	7	16	35	41	45	2	6	12	15	18(35)
A-8	1:1.25	P	16	46	47	47		4	33	39(72)	37	
		E	7	17	37	38		2	7	11(25)	11	
A-9	1:1.5	P	15	45	47	47	48	2	41	43(80)	40	38
		E	10	23	40	46		2	9	15	28(53)	
A-10	1:1.75	P	18	47	46	46	48	4	36	37	42(80)	39
		E	12	31	46	45	48	2	9	24	23	26(47)
A -11	1:2	P	19	41	40	45	48	3	24	30	37	45(82)
		E	9	30	45	41	44	2	12	22(43)	15	15

The legend is the same as in Table II.
* Underlined figure shows the result obtained by paper chromatography.

salt employed. Thus in order to choose the most favorable salt, the effect of media A-1~A-6, which contain ammonium fumarate, ammonium chloride, ammonium phosphate, ammonium nitrate, ammonium sulfate and urea, respectively, was compared.

As shown in Table II, it was found that the effect of ammonium salts differs according to the organism employed. Namely, for *Pseudomonas fluorescens* no appreciable accumulation of aspartate was observed except in the case of ammonium fumarate. On the other hand, ammonium fumarate, ammonium chloride and ammonium phosphate were utilized by *Escherichia coli* and accumulation occurred, e.g., 35 mg/ml of aspartate was formed in the case of ammonium chloride on 9 days culture.

In the case of medium A-5 containing ammonium sulfate, though fumarate was appreciably consumed, the accumulation of aspartate was poor. Further it was found that medium A-6 containing urea was unfavorable for growth of organisms and aspartate formation.

II) Effect of Molar Ratio of Fumarate and Ammonia

Although aspartate was formed stoichiometrically from fumarate: ammonium ion=1; 1, the ratio was changed (1 : 0.75∼2) by using media A-1 and A-7∼A-11 in order to disclose the optimum ratio for aspartate formation.

The results, shown in Table III, indicate the tendency that consumption of fumarate and accumulation of aspartate in *Pseudomonas fluorescens* proceed faster than that of *Escherichia coli*, and yield of aspartate is also better in the former case.

For *Pseudomonas fluorescens*, a fumarate : ammonium ion ratio of 1 : 1.5∼2 gave good reasults and approximately 80% conversion rate was obtained. In *Escherichia coli* the ratio 1 : 1.5 gave a preferable result, though the yield of aspartate was low. It is quite natural that both organisms exhibited poor aspartate formation in medium A-7 which ratio was 1 : 0.75.

III) Effect of pH

For the purpose of investigating the effect of pH on the formation of aspartate, the pH of the culture was controlled as follows: starting from initial pH 7.0, pH of the culture was adjusted to pH 6.0, 7.0 and 8.0 at intervals of 24 hrs. As for the control, experiments without pH adjustment and addition of 1% calcium carbonate (medium A-12) to medium A-1 were also carried out. All experiments were conducted with medium A-1 except in the case of medium A-12 at 30°C by shaken culture.

As shown in Table IV, pH adjustment at 24 hr-intervals was found to be useless to maintain the designated pH and the pH of cultures shifted to alkaline side.

When the pH was adjusted, generally much formation of other amino acids from aspartic acid and less accumulation of aspartate were observed than that of without pH adjustment. For instance, 6 days' culture of *Pseudomonas fluorescens* adjusted to pH 7.0 yielded more glutamic acid (ca 15 mg/ml) than aspartic acid.

Although the addition of calcium carbonate showed no appreciable effect in the case of *Pseudomonas fluorescens*, it was effective in *Escherichia coli* to prevent the lowering of pH at the initial stage of fermentation, and resulted in a higher yield of aspartate.

VI) Effect of addition of glucose

It is quite probable that the fermentation products were influenced by the addition of glucose to the medium which contains fumarate as the main carbon source, so that the effect of glucose was studied by media A-13∼A-17, which contained 0, 0.5, 2, 5, and 10% glucose, respectively.

As shown in Table V, the best result was obtained in both strains when glucose was not added, in which case better accumulation of aspartate was observed and no amino acid other than aspartic acid was formed.

An increase in glucose concentration of the medium retarded the consumption of fumarate and formation of aspartate. This phenomenon may be the result of retardation of growth of the organism due to prolongation of the period

TABLE IV. EFFECT OF pH

Medium	pH	Strain	pH						Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
			Days of culture													
			1	2	3	4	6	9	2	4	6	9	2	4	6	9
A-1	6.0	P	6.8	7.4	8.6	8.4	8.2	8.4	9	39	47	48	2	8	28(52)	25
		E	5.4	8.0	8.4	8.2	8.6	8.8	4	20	43	46	2	4	6	8(15)
A-1	7.0	P	6.8	8.0	8.6	8.8	9.0	9.2	19	47	47	48	3	14(27)	10	7
		E	5.4	8.2	8.6	8.4	8.6	9.0	12	28	41	46	2	7	10(21)	8
A-1	8.0	P	6.8	8.6	9.0	8.6	8.6	9.0	35	47	46	47	5	23	30(57)	19
		E	5.4	8.4	8.6	8.6	8.6	9.0	10	34	45	46	2	8	12	10 : 20(38)
A-1	control	P	6.8	8.4	8.8	9.0	9.2	9.0	16	47	46	46	3	35(66)	35	31
		E	5.4	8.0	8.4	8.8	8.6	8.8	7	16	45	41	2	6	12	15(31)
A-12	$CaCO_3$	P	6.8	8.6	8.6	8.8	8.8	9.2	15	46	47	47	4	36	37(68)	31
		E	6.4	8.4	8.8	8.8	8.8	9.0	20	44	45	46	3	20	32(62)	29

The legend is the same as in Table III.

TABLE V. EFFECT OF ADDITION OF GLUCOSE

Medium	Glucose	Strain	pH				Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
							Days of culture							
			2	4	6	9	2	4	6	9	2	4	6	9
A–13	0	P	8.8	9.4	9.2	9.2	18	40	44	47	4	16	23	28(52)
		E	8.0	8.8	8.8	9.2	24	38	46	47	14	25	29	40(74)
A–14	0.5	P	8.6	9.4	8.8	9.4	23	44	46	48	3	19	28(53)	24
		E	8.4	9.0	9.2	9.4	20	37	45	48	2	15	24(47)	14 : 25
A–15	2.0	P	8.4	9.0	8.8	9.4	12	47	44	46	2	17	25	30(57)
		E	8.2	9.0	9.4	9.0	28	43	48	48	2	12	20(36)	12 : 20
A–16	5.0	P	7.0	8.6	8.8	9.2	8	40	46	48	2	1	4	10 : 15(27)
		E	6.0	7.4	8.6	8.8	8	33	47	46	1	1	1	1
A–17	10.0	P	6.8	7.4	8.6	9.2	8	41	42	46	2	1	1	2
		E	6.2	6.8	8.6	8.8	13	37	45	45	1	1	6	11(21)

The legend is the same as in Table III.

TABLE VI. EFFECT OF CONCENTRATION OF FUMARATE

Medium	Fumarate	Strain	pH			Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
						Days of culture							
			2	4	6	2	4	6	9	2	4	6	9
A–18	2	P	9.2	9.4	9.4	15	15	16	16	0	0	0	1
		E	8.6	8.8	9.0	5	17	17	18	2	11	13	14
A–12	5	P	8.6	9.0	9.2	22	47	47	47	5	30(56)	30	30
		E	8.6	9.0	9.0	31	48	47	48	16	34	38(70)	38
A–19	10	P	6.6	7.8	7.8	7	11	15	15	4	4	2	2
		E	6.8	7.8	7.8	10	14	18	26	5	8	9	18
A–20	20	P	6.4	7.8	7.8	15	20	20	32	12	14	11	10
		E	7.4	7.8	7.8	20	27	35	40	10	13	12	13

The legend is the same in Table III.

of low pH at the initial stage of fermentation by the addition of glucose. Also, on prolonged incubation in the medium containing glucose, formation of other amino acids such as glutamic acid and alanine was observed.

V) Effect of Fumarate Concentration

Cultures in media A-12 and A-18∼A-20, which contained 2.5, 10 and 20% fumarate, respectively, were investigated in order to find the suitable fumarate concentration in the medium for aspartate accumulation.

From the results, shown in Table VI, it is obvious that 5% fumarate in the medium gives the best result for the both strains tested.

An increase in fumarate concentration exceeding 5% retarded consumption of fumarate and resulted in poor aspartate accumulation.

VI) Effect of Organic Nitrogen Source

Organic nitrogen source in the medium may be useful not only for the growth of organism but also for the acceleration of enzyme formation concerning with aspartate production.

Therefore the effect of various organic nitrogen compounds was investigated by the experiments on media A-21∼A-27 which contain casein hydrolysate, corn steep liquor, peptone, yeast extract and meat extract. These organic nitorogen substances are added to the media at the same concentration, i. e., 0.1% as N. As a control run, culture on medium A-12, which contains 0.03% organic nitrogen compounds as N, was also conducted.

The results obtained are shown in Table VII. Comparatively higher yields of aspartate were obtained by the media containing peptone, yeast extract and meat extract, e. g., 6 days' culture of *Escherichia coli* on medium A-23 containing peptone yielded aspartate in 42 mg/ml concentration.

In the case of corn steep liquor, though

TABLE VII. EFFECT OF ORGANIC N SOURCE

Medium	Organic N Source	Strain	Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
			Days of culture							
			2	4	6	9	2	4	6	9
A-21	Casein hydrolysate	P	7	34	40	43	2	15	20	23(47)
		E	22	48	46	46	3	8	8	10(19)
A-22	Corn steep liquor	P	42	40	39	39	15	23(50)	21	20
		E	43	47	47	46	7	14	16(30)	15
A-23	Peptone	P	18	45	44	42	2	35	34	37(77)
		E	15	48	48	48	5	37	42(77)	39
A-24	Yeast extract	P	40	43	43	41	24	35(72)	33	27
		E	7	31	44	44	2	10	32	34(68)
A-25	Meat extract	P	17	43	45	46	7	34(68)	28	35
		E	33	39	46	45	12	15	32(61)	23
A-26	Meat extract +Peptone	P	36	47	45	44	7	28	28	35(69)
		E	28	47	47	47	4	32	33	36(67)
A-27	Yeast extract +Peptone	P	35	45	45	45	7	33(64)	30	30
		E	32	45	44	48	4	24	28(55)	26
A-12*	Yeast extract +Peptone	P	17	46	46	45	2	28	28	31(60)
		E	14	46	46	47	2	29	31	36(67)

The legend is the same as in Table III.

* Medium A-12 contains organic substances in 0.03% as N, that is, one-third of Medium A-27.

TABLE VIII. COMPARISON OF WILD STRAIN AND MUTANTS OF *E. coli.* K-12

Medium	Strain	pH					Fum. cons. (mg/ml)				Asp. form. (mg/ml)			
		Days of culture												
		1	2	4	6	8	2	4	6	8	2	4	6	8
A-28	Wild	7.0	8.2	8.6	8.6	8.8	7	18	36	48	1	11	30	41(74)
A-29		7.4	8.4	8.6	8.8	8.8	10	28	48	48	2	19	37	39(71)
A-28	Ki-1023	7.0	8.2	8.4	8.8	8.8	11	34	48	47	2	20	40	44(81)
A-29		7.4	8.4	8.6	8.8	8.8	18	48	47	48	5	34	44	45(82)
A-28	Ki-1282	7.2	8.2	8.6	8.4	8.6	1	17	36	48	1	10	24	34(62)

The legend is the same as in Table III.

fumarate was consumed more rapidly conversion to aspartate was low as in the case of casein hydrolysate. As shown in experiments on media A-21 and A-22, a higher concentration of organic nitrogen source resulted in more rapid consumption of fumarate. However, amounts of aspartate accumulated in respective cultures showed no appreciable difference.

VII) Comparison of Wild Strain and Mutants of *Escherichia coli* K-12

Mutants such as Ki-1023 (methionine requiring mutant) and Ki-1282 (threonine requiring mutant) derived from *Escherichia coli* K-12, were compared with parent strain on their activity forming aspartate by employing media A-28 and A-29.

From the results, shown in Table VIII, it is ascertained that Ki-1023 is the most suitable strain for aspartate production. As shown in experiments with Ki-1023, although the addition of calcium carbonate (medium A-29) accelerated the consumption of fumarate and formation of aspartate at the initial stage of fermentation than in case without addition (medium A-28), the maximum amounts of formed aspartate were almost the same on both media.

In another mutant, Ki-1282, accumulation of aspartate was less than that of the wild strain.

VIII) Effect of Temperature and Shaking

All previous experiments were carried out at 30°C with reciprocal shaking. To investigate the effect of incubation temperature and shaking on the accumulation of aspartate, shaking and stationary culture, and partially stationary

TABLE IX. EFFECT OF TEMPERATURE AND SHAKING

Temp. (°C)	Methods of Culture	Medium	Strain	Fum. cons. (mg/ml) Days of culture				Asp. form. (mg/ml)			
				2	4	6	8	2	4	6	8
30	Shaking	A-28	P	27	46	47	46	3	30	32	35(67)
		A-29	E	17	43	48	45	3	26	31	45(89)
	Stationary*	A-28	P	12	45	48	48	1	33	40(73)	37
		A-29	E	17	47	48	47	3	40	38	48(89)
	Stationary	A-28	P	3	12	36	47	0	8	19	34(63)
		A-29	E	8	39	48	46	2	20	38	42(80)
	Shaking	A-28	P	37	46	46	46	14	26	29(55)	27
		A-29	E	35	48	49	48	29	41(75)	17 : 30	3 : 20
37	Stationary*	A-28	P	28	47	48	47	14	43(80)	42	35
		A-29	E	33	49	48	47	17	41	48(87)	36
	Stationary	A-28	P	4	27	46	45	2	19	38	42(82)
		A-29	E	17	48	48	48	7	37	46(84)	45
	Shaking	A-32	E	2	13	44	45	3	3	16 : 20	19 : 25(48)
	Stationary*			5	18	32	46	2	6	22	35(67)

Stationary* indicates partially stationary culture in which shaking was done for initial one day. Other legend is the same as in Table III.

culture, i. e., stationary culture after the shaking at the initial day, were carried out at 30°C and 37°C. For *Pseudomonas fluorescens*, medium A-28 was employed and for *Escherichia coli* media A-29 and A-32 were used to compare ammonium fumarate and ammonium chloride.

As shown in Table IX, the consumption of fumarate and formation of aspartate were promoted by the incubation at 37°C than that of incubation at 30°C. No significant difference was found for both strains between stationary and shaking cultures at 30°C. Although, at 37°C stationary culture was evidently advantageous than shaking culture for *Pseudomonas fluorescens*, and *Escherichia coli* also showed the same tendency.

Of the tested cultural cooditions, partially stationary cultures in which shaking was done for the initial day gave the best result for both tested strains. Namely, shaking at the initial stage of fermentation favors the growth of organism, and later stationary incubation is considered advantageous for formation and accumulation of asparatate.

In the previous experiment on the effect of ammonium salts, ammonium chloride gave a better result than ammonium fumarate for *Escherichia coli*. Although, as revealed by the present experiment, the addition of calcium carbonate made the medium containing ammonium fumarate (medium A-29) advantageous over the medium employing ammonium chloride.

Cultures under Optimum Conditions

Since the most favorable cultural conditions for aspartate accumulation were investigated by the above fermentation experiments, practical formation of aspartate was carried out under optimum conditions. For sake of comparison, shaking culture was also conducted. The results are shown in Figs. 1 and 2.

In the case of *Pseudomonas fluorescens*, fumarate was consumed in 3 days in both cultures, and the attained conversion to aspartate was higher than 95% on the partially stationary culture. In case of shaking culture, conversion to aspartate was worse, just as previously observed and also showed a tendency to decrease formd aspartate by prolonged incubation.

In the case of *Escherichia coli*, fumarate was consumed in 3 days and the maximum conversion to aspartate was attained in 4 days' culture, e. g. the maximum yield of aspartate almost reached the theoretical amount, 56 mg/ml by partially stationary culture.

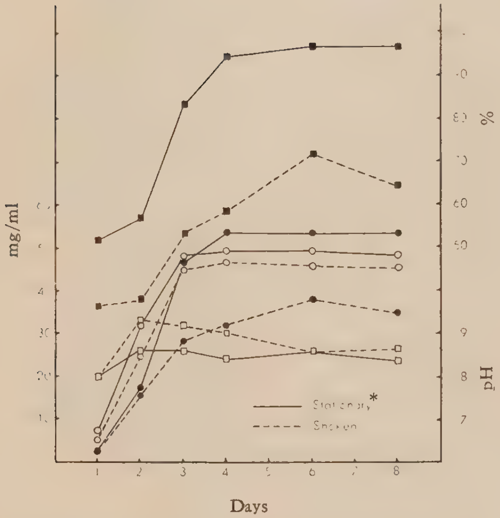

FIG. 1. Fermentation by *Pseudomonas fluorescens*
Fermentation was carried out with medium A-30 at 37°C.

□ pH
○ Fum. cons.
● Asp. form.
■ Conv. ratio

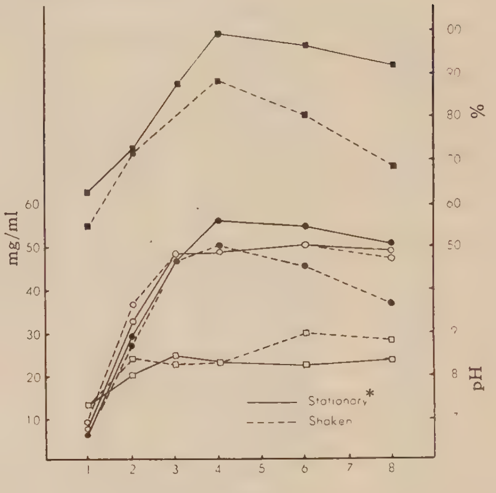

FIG. 2. Fermentation by *Escherichia* K-12 Ki-1023
Fermentation was carried out with medium A-31 at 37°C.

□ pH
○ Fum. cons.
● Asp. form.
■ Conv. ratio

With the above 4 days' culture, the practical isolation of L-aspartic acid was carried out. From the culture broth, cells and calcium carbonate were removed by centrifugation. The supernatant solution was made up to pH 5.0 and boiled for 30 minutes. After decolorization with active carbon, the filtrate was codensed in vacuo. After it was adjusted to pH 3.0, the condensed filtrate was cooled, and crystallized L-aspartic acid was collected by filtration. Recrystallization from water yielded colorless fine plates, 50 mg/ml (87%), m. p. 250°C (decomp.), $[\alpha]_D^8=+25.1°$ (C=8, 6N HCl). *Anal.* Calcd. for $C_4H_7NO_4$: C, 36.09; H, 5.26; N, 10.53. Found: C, 36.13; H, 5.29; N, 10.13.

The presence of amino acids other than aspartic acid was not found in the sample obtained.

SUMMARY

A number of organisms were screened to test activity of accumulating aspartate from fumarate and ammonium salts and, *Pseudomonas fluorescens* 6009-2 and mutant Ki-1023 derived from *Escherichia coli* K-12 by ultraviolet radiation were chosen as the suitable strains.

By employing these strains various cultural conditions suitable for the production of L-aspartic acid were investigated and, the results obtained are as follows:

a) Ammonium fumarate is the most favorable as ammonium salts.

b) On the molar ratio of fumarate and ammonium ion in the medium, the ratio 1 : 1.5~2.0 gives the best result.

c) On the effect of pH, culture without pH adjustment starting from initial pH 7.0, is preferable for *Pseudomonas fluorescens* than in case of pH adjustment during incubation, while on *Escherichia coli* the addition of calcium carbonate results in better accumulation of aspartate.

d) The addition of glucose causes formation of amino acids other than aspartic acid and lowers the yield of aspartate.

e) On the concentration of fumarate in the

medium, 5% gives the best result.

f) Of the various organic nitrogen compounds in the medium, peptone is most favorable.

g) Of the wild strain and mutants of *Escherichia coli* K-12, Ki-1023 is ascertained to be the most suitable strain.

h) Incubation at 37°C accelerates formation of aspartate than incubation at 30°C, though there is no difference of the attained conversion rate.

i) On the influence of shaking, partially stationary culture, that is, after shaking at initial day stationary culture is done, enables better result than stationary or shaking culture throughout the period of incubation.

Under the optimum conditions revealed by the above experiments, practical formation of aspartate was carried out, and formation over 95% conversion rate from consumed fumarate was attained by both strains. The formed aspartate was readily isolated from the culture broth and identified as L-aspartic acid. This method is considered to be an advantageous procedure for the production of L-aspartic acid.

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Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria

Part X. Some Properties of Pentose Isomerase by Hetero-type of Lactic Acid Bacteria*

By Kei YAMANAKA

Laboratory of Microbiology, Faculty of Agriculture, Kagawa University, Kagawa

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The pentose isomerase of heterofermenters was found to have specific action on the substrate. Only L-arabinose was isomerized by L-arabinose isomerase and no inhibitory effect was demonstrated by the addition of D-isomer, and xylose isomerase acted on D-xylose, and L-xylose did not inhibit such activity. No ketose from glucose, mannose, galactose, L-xylose, D-arabinose, D-ribose and mannitol was produced by these heterofermenters. The equilibrium constants for D-xylose- and L-arabinose isomerases were 20~28% and 13~16% respectively, and this equilibrium was shifted to ketopentose in the presence of borate. Michaelis constants were found to be 2×10^{-2} M for D-xylose isomerase and $5\sim10\times10^{-2}$ M for L-arabinose isomerase.

INTRODUCTION

In the previous paper, the D-xylose- and L-arabinose-isomerases by the hetero-type of lactic acid bacteria (heterofermenter) have already been reported[1]. Since the products from D-xylose or L-arabinose were isolated by a Dowex-1 borate resin column and identified as xylulose and ribulose respectively, it was suggested that the initial step of pentose fermentation by these heterofermenters was its isomerization in coincidence with the results by homofermenter already shown by Horecker et al.[2,3].

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* Presented before the annual meeting of the Agricultural Chemical Society of Japan at April 8th, 1959.

[1] K. Yamanaka, This Bulletin, **22**, 299 (1958).

[2] B. L. Horecker, E. C. Heath, J. Hurwitz and A. Ginsberg, *Federation Proc.*, **16**, 198 (1957).

[3] B. L. Horecker, E. C. Heath, J. Hurwitz, Y. Takagi and D. P. Burma, *Proc. Interntl. Sym. on Enz. Chem.*, 99 (1957).

In this paper, effect of cultural condition, effect of D, L-isomer on pentose isomerase activity and some chemical properties of pentose isomerases demonstrated by eight strains of heterofermenters will be described. These enzymatic preparations showed similar properties, so no difference was demonstrated among the strains used here. Further studies on the production of pentose isomerase are the subject of the following paper[4].

MATERIALS AND METHODS

Materials D-Xylose was a commercial product of E. Merck and L-xylose a Mann Research Laboratory, Inc. product. L- and D-Arabinoses were obtained from the above Laboratories.

Microorganisms Eight strains of heterofermenters illustrated as follows were used: *L. fermentum*, *L. fermenti* ATCC 9338, *L. brevis* ATCC 8281, *L. pentoaceticus* ATCC 368, *L. gayonii* ATCC 8289, *L. mannitopoeus*, *L. lycopersici* and *L. buchneri*.

Culture Bacteria were cultured at 37°C for 20 hours in 0.8 L of the medium containing 1% peptone, 1% yeast extracts, 1% sodium acetate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$ and 1% pentose. Sugars were sterilized separately as a concentrated solution and added to sterile medium just before inoculation.

L-Arabinose isomerase was prepared from the cells grown on a medium containing L-arabinose as a single carbon source, and D-xylose isomerase was prepared from D-xylose-grown cells. Cells were harvested by centrifugation, and crude cell-free extracts were prepared by grinding the cells with twice their weight of levigated alumina in a chilled mortar at 0°C for five minutes, extracted with a desired volume of chilled distilled water, and then centrifuged in the cold at 13,000 g for thirty minutes, a clear supernatant was used as crude extracts. This extracts was treated with protamine sulphate to remove the nucleic acid.

Enzymatic Assay and Determinations The reaction system was composed of 10 μM of $MgCl_2$, 50 μM of tris-(hydroxymethyl)-amino methane buffer at pH 7.4, 20 μM of pentose, 20 μM of sodium fluoride and cell-free extracts. The total volume was adjusted to 3.0 ml with distilled water and incubated at 37°C. Protein and ketopentoses were determined as described in the previous paper[1].

RESULTS

Substrate Specificity

The cell-free extracts of *L. gayonii* grown on glucose medium, and of *L. lycopersici* grown on glucose or D-xylose medium were found to be specific in acting only on D-xylose or L-arabinose (Table I). No ketose was formed from glucose, mannose, galactose, L-xylose, D-arabinose, D-ribose, rhamnose, and mannitol. These extracts were shown to have high specificity for the substrate reacting only on D-xylose and L-arabinose in this experimental conditions.

TABLE I. SPECIFICITY OF SUBSTRATE

Ketose formed (μM, 37°C, 30 min.)

Organisms	*L. gayonii*	*L. lycopersici*		*L. fermentum*
C-source	glucose	glucose	xylose	mannose
extracts used (protein, mg)	1.32	0.82	1.45	1.58
glucose	0	0	0	0
mannose	0	0	0	0
galactose	0	0	0	0
D-xylose	0.45	0.37	4.53	2.5
L-xylose	0	0	0	0
L-arabinose	2.4	1.9	0.78	0
D-arabinose	0	0	0	0
D-ribose	0	0	0	0
rhamnose	0	0	0	0
mannitol	0	0	0	0

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[4] K. Yamanaka, This Bulletin, **24**, 310 (1960).

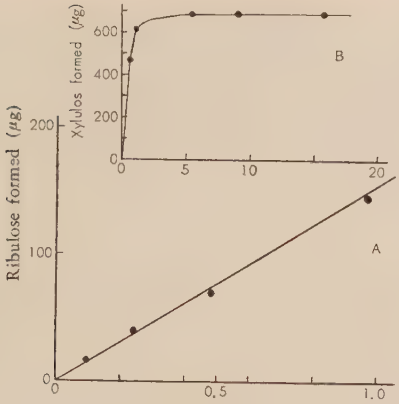

FIG. 1. Effect of Enzyme Concentration

A. L-Arabinose isomerase of *L. gayonii* (37°C, 15 min.)
B. D-Xylose isomerase of *L. gayonii* (37°C, 30 min.)

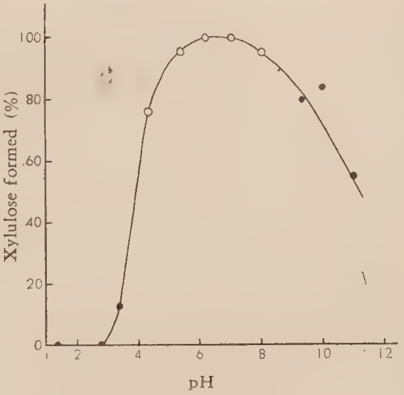

FIG. 2. Optimum pH

D-Xylose isomerase of *L. pentoaceticus* (Protein, 0.42 mg, 37°C, 10 min.)
○—○ Phosphate buffer
●—● Glycine buffer

Similar results were also obtained from cells of *L. fermentum* grown on mannose. As will be noted later⁴⁾, this bacterium did not ferment L-arabinose, but xylose isomerase was found to be active.

Effect of Enzyme Concentration and pH

A proportional of enzyme (within 1.0 mg of protein) to the reaction product was obtained as will be seen in Fig. 1. With more increased concentration of enzyme, no further increase of ketose formation was observed. The effect of pH for xylose isomerase of *L. pentoaceticus* was assayed, and maximum activity was observed at a relatively wide range of pH 5.4 to 8.0. Similar results were also obtained with other enzyme preparations.

Effect of Shaking Culture

L. fermentum was cultured statically on glucose as the carbon source at 37°C for twenty hours, or on shaker at 35°C for eighteen hours, and the gaseous metabolism of sugars by the intact cells were determined with a Warburg ma-

TABLE II. OXYGENE UPTAKE FROM SUGARS BY INTACT CELLS

Medium	C-Source	pH	Culture (hr.)	Cell used (mg/0.5 ml)	O_2 Uptake (µl/2 hrs.)						
					D-X	L-A	D-R	G	F	M	GA
B*	glucose	5.4	static (40)	11.7	0	0	0	16	9	7.5	—
			shaking (18)	10.9	0	0	0	5	—	—	—
A*	glucose	7.0	static (40)	4.8	0	0	0	0	0	0	0
			shaking (18)	3.3	5	2	9	230	160	185	112
		5.4	static (40)	2.3	0	0	0	0	0	0	2
			shaking (18)	10.2	6	0	19	227	193	232	105
A*	xylose	5.4	shaking (40)	1.12	0	0	0	0	0	0	0

* Medium A was composed as follows: 1% peptone, 1% yeast extracts, and 0.5% K_2HPO_4 (pH 7.0). Medium A was acidified to pH 5.4 by the addition of conc. HCl.

* Medium B was composed as follows: 1% peptone, 1% yeast extracts, 1% sodium acetate, 0.02% $MgSO_4$, 0.001% NaCl, 0.001% $FeSO_4$, and 0.001% $MnSO_4$.

The following abbreviations are used; D-X, D-xylose; L-A, L-arabinose; D-R, D-ribose; G, glucose; F, fructose; M, mannose; GA, gluconate.

TABLE III. EFFECT OF CULTURE
(*L. fermentum*, C-source mannose)

Medium*	Culture	Protein (mg)	D-Xylose isomerase activity (μg/mg of protein)
A	static	0.46	118
	shaking	1.02	224
B	static	4.45	368
	shaking	3.05	169

* Compositions of media A and B see in Table II.

nometer. These intact cells of *L. fermentum* showed no oxygen consumption and no carbon dioxide evolution even by the cells cultured in shaking on a xylose medium. Moreover, the activity of pentose isomerase was observed with the cell-free extracts prepared from the cells grown on mannose obtained from both static and shaking cultures as seen in Table III.

Effect of D, L-Isomers

As will be seen in the following paper[4], D-arabinose was not fermented by heterofermenters, even when it fermented L-arabinose, and D-arabinose and L-xylose were not isomerized by enzyme preparations of these organisms. Furthermore, D-arabinose did not reveal any inhibitory effect on L-arabinose isomerase activity. Table IV represents three cases of the effect of D-arabinose on L-arabinose isomerases of *L. gayonii*, *L. brevis* and of *L. lycopersici*. These effects were also found not only with D-arabinose on L-arabinose isomerase but also with L-xylose on D-xylose isomerase. The addition of L-xylose (0 to 200 μM) did not affect any inhibition of D-xylose isomerase activity. The same results were obtained in many cases with xylose isomerases of *L. buchneri* and of *L. mannitopoeus* listed in Table V.

TABLE IV. EFFECT OF D-ARABINOSE ON L-ARABINOSE ISOMERASE

D-Arabinose added (μM)	L-Ribulose formed (μM)		
	*L. gayonii**	*L. brevis***	*L. lycopersici****
0	2.91	2.35	2.61
20	2.94	2.32	2.45
50	2.94	2.35	2.49
100	2.83	2.37	2.44
200	2.72	—	2.62

* *L. gayonii*, L-arabinose grown, protein 1.5 mg, 37°C, 30 min.
** *L. brevis*, 〃 〃 , 〃 1.32 mg, 〃 , 30 min.
*** *L. lycopersici*, 〃 〃 , 〃 1.48 mg, 〃 , 10 min.

TABLE V. EFFECT OF L-XYLOSE ON D-XYLOSE ISOMERASE

L-Xylose added (μM)	D-Xylulose formed (μM)	
	*L. buchneri**	*L. mannitopoeus***
0	3.82	4.56
20	3.87	4.30
50	4.08	4.32
100	3.60	4.48
200	3.90	4.30

* *L. buchneri*, D-xylose grown, protein 1.47 mg, 37°C, 30 min.
** *L. mannitopoeus*, 〃 〃 , 〃 1.86 mg, 〃 , 15 min.

TABLE VI. EQUILIBRIUM OF ISOMERASE ACTION

Organism	Per cent of ketopentose	
	D-Xylose isomerase	L-Arabinose isomerase
*L. fermentum**	27.2**	—
*L. fermenti**	19.5	—
*Leuc. mesenteroides**	8.5	—
L. brevis	21∼24 → 98***	13.4** → 73.5***
L. pentoaceticus	28.0 → 96.5	13.3 → 75.4
L. gayonii	21∼24 → 84	16.5 → 85
L. mannitopoeus	24 → 97	14.5 → 74
L. lycopersici	23.5 → 98	15.3 → 82
L. buchneri	23.3 → 98	11.7 → 64.5

* Addition of borate showed no or slightly inhibitory effect.
** Borate was not added.
*** Twenty micromoles of borate was added.

Equilibrium and Michaelis Constants

The accumulation of ketopentose was examined in prolonged incubation, and the ketose yield percentage was expressed as the equilibrium constant. This reaction was reversible and when L-ribulose or D-xylulose was incubated with the enzymatic preparation, the same equilibrium constant was noted. These constants for D-xylose isomerase were found to be 20～28 per cent, and for L-arabinose isomerase 13～17 per cent. The addition of borate displaced this equilibrium strongly in favor of the ketose. The Michaelis constants based on the Lineweaver-Burk's equation were also determined. D-Xylose isomerase showed $2\sim3\times10^{-2}$ M and its constant for L-arabinose isomerase was somehow higher than that for D-xylose isomerase. A typical result is shown in Fig. 3, and summarized in Table VII.

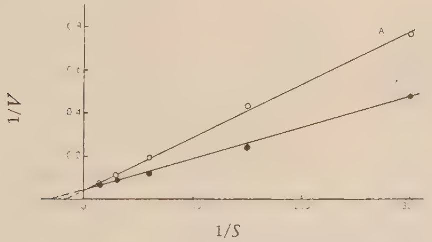

FIG. 3. Effect of Substrate Concentration for Pentose Isomerase of *L. mannitopoeus*

A. L-Arabinose isomerase, X. D-Xylose isomerase

TABLE VII. K_M VALUES FOR PENTOSE ISOMERASE

Organism	Michaelis constant ($\times 10^{-2}$M) D-Xylose isomerase	L-Arabinose isomerase
L. pentoaceticus	2.08	4.76
L. gayonii	2.16	5.62
L. mannitopoeus	3.21	10.33
L. buchneri	2.47	8.77
L. brevis	—	9.90

DISCUSSION

It has been already known that pentose is fermented to each equal mole of lactate and acetate without carbon dioxide evolution nor oxygen uptake by homo- and heterofermenters under an anaerobic condition. In shaking culture, xylose was dissimilated in a manner similar to that of the anaerobic condition by *L. fermentum* and by *L. xylosus*[5]. As only a trace or no oxygen uptake from D-xylose, D-ribose and L-arabinose was demonstrated by the resting cells of *L. fermentum* grown on shaking culture, and the activity of xylose isomerase does not affect both static and in shaking cultures, it can be clearly stated that the oxidative pathway for pentoses such as D-ribose oxidation by *L. plantarum*[6] could not be demonstrated in *L. fermentum*, and that xylose is first isomerized to xylulose in both anaerobic and aerobic conditions.

D-Xylose- and L-arabinose isomerases of heterofermenters showed highly substrate specificity exclusively on D-xylose or L-arabinose, and any inhibitory effects were not demonstrated by the addition of these stereoisomers on isomerase activity. Thence, it can be presumed that these isomers do not show affinity to the isomerase.

The percentage of ketopentose obtained from aldopentose by the action of isomerase of some bacteria has been observed to be as follows: 14 per cent at 38°C by L-arabinose isomerase of *L. plantarum* (*L. pentosus*)[7], 15 per cent by D-arabinose isomerase of *E. coli*[8], and for D-xylose isomerase, 13.5[9] and 18[10] per cent by *L. pentosus* (*L. plantarum*), and 16 per cent by *Pseud. hydrophila*[11] and also by *Pasteurella pestis*[12]. As shown in the present paper, for L-arabinose isomerase of heterofermenters, the content of L-ribulose at equilibrium was found to be 13～16 per cent, while on the other hand, D-xylose isomerase of heterofermenters showed a somehow higher equilibrium value than the isomerase of other bacteria.

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[5] H. Katagiri and K. Imai, This Bulletin, **19**, 15 (1955).

[6] S. Fukui and A. Ôi, *J. Gen. Appl. Microbiol.* (Japan), **4**, 120 (1958).

[7] E. C. Heath, B. L. Horecker, P. Z. Smyrniotis and Y. Takagi, *J. Biol. Chem.*, **231**, 1031 (1958).

[8] S. S. Cohen, *J. Biol. Chem.*, **201**, 71 (1953).

[9] S. Mitsuhashi and J. O. Lampen, *ibid.*, **204**, 1011 (1953).

[10] D. P. Burma and B. L. Horecker, *ibid.*, **231**, 1053 (1958).

[11] R. M. Hochster and R. W. Watson, *Arch. Biochem. Biophys.*, **48**, 120 (1954); *J. Am. Chem. Soc.*, **75**, 3284 (1953).

[12] M. W. Slein, *J. Am. Chem. Soc.*, **77**, 1663 (1955).

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 310~317, 1960]

Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria

Part XI. Relation between Carbon Source and Pentose Isomerase Production

By Kei YAMANAKA

Laboratory of Microbiology, Faculty of Agriculture, Kagawa University, Kagawa

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Six strains of homofermenters produced L-arabinose isomerase in only the L-arabinose-medium, and with these bacterial cells which were obtained from the medium containing glucose, fructose or mannose, any activity of this enzyme was not pointed out. Moreover the L-arabinose isomerase of six strains of heterofermenters and of *Pediococcus lindneri*, and also D-xylose isomerase of six strains of heterofermenters and of *L. xylosus* are produced even in glucose, fructose, mannose, galactose or pentose-media. The amounts of D-xylose isomerase of *L. fermentum* varied according to the kind of carbon source of the growing culture. This xylose isomerase is produced abundantly in the cells grown on mannose, L-arabinose and D-xylose-media. Glucose, galactose, maltose, sucrose, and gluconate do not show any stimulating effect on the production of xylose isomerase.

INTRODUCTION

Our knowledge on the production of pentose isomerase by microorganisms is still obscure, and only a few reports concerning this problem are available in which it is suggested that its production is adaptively due to the substrate of growing culture. These data will be briefly summarized in the discussion section. On the other hand, Karström[1] in 1938 has already shown that the fermentation of pentose by some bacteria is constantly proceeded, while the ability of fermentation of pentose by another bacteria is not found to be constant. In the latter, pentose was dissimilated exclusively by the cells grown on pentose. Karström had thus been suggested to introduce two categories for enzyme production, constitutive and adaptive. Some workers have shown that the L-arabinose- and D-xylose isomerases of homolactic acid bacteria are produced adaptively only on each substrate.

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[1] H. Karström, *Erg. d. Enzymforsch.*, 7, 350 (1938).

Aside from these recognitions, the production of these isomerases by another type of bacteria-heterofermenters which has not yet been discussed, was undertaken with the object of obtaining the more precise results of this problem.

For this purpose, the cultures were tested with a medium containing a single carbon source among various kinds of sugars, and the change of activities of isomerase were assayed using its cell-free extracts. As it will be shown, the production of pentose isomerase by most strains of heterofermenters was always found even from the medium which did not contain pentose. In contrast, the presence of these enzymes in most strains of homofermenters was only demonstrated in the pentose-containing medium. Hence it was strongly suggested that the heterofermenters produce this enzyme constitutively, while the homofermenters produce it adaptively.

METHODS

Microorganisms For heterofermenters, except listed in the previous paper[2], about ten strains of bacteria were

TABLE I. ACID PRODUCTION FROM PENTOSES

Organism	D-Xylose	L-Arabinose	D-Arabinose	Glucose
L. brevis, ATCC 8281	2.7	2.4	0.06	0.9
L. pentoaceticus, ATCC 368	1.9	2.7	0.06	0.6
L. mannitopoeus	1.6	2.7	0.04	1.9
L. lycopersici	3.5	3.9	0.07	1.2
L. gayonii, ATCC 8289	7.7	4.6	0.20	0.5
L. buchneri	3.0	4.6	0.14	0.0
L. fermentum β	2.9	0.2	0.07	3.9
L. fermenti, ATCC 9338	3.9	0.7	—	3.2
Leuc. mesenteroides α	6.0	2.7	0.0	3.1
Leuc. mesenteroides, ATCC 8042	3.3	3.6	0.20	3.4
Leuc. mesenteroides, ATCC 9135	3.7	5.5	0.03	5.8
Leuc. dextranicum, ATCC 8086	0.3	0.2	—	2.5
Leuc. citrovorum, ATCC 8081	0	0.6	—	1.8
L. delbrueckii	0.2	0.1	—	6.5
L. lactis	0.3	0.2	—	5.1
L. helveticus, ATCC 335	0	0	—	2.5
L. acidophilus	0.6	0.5	—	6.9
L. bulgaricus	0.6	0.4	—	8.2
L. casei	0	0.3	—	6.8
L. plantarum	0.1	2.0	0.07	6.5
L. plantarum, ATCC 8008	0.3	1.9	0.22	4.9
L. arabinosus, ATCC 8044	1.1	6.6	0.07	7.5
L. cucumeris	0.6	2.6	0.05	3.8
L. saké	0	3.3	0	2.8
L. wortmannii	0	2.5	0	4.3
L. xylosus	7.4	0.5	—	6.4
L. leichmannii, ATCC 4797	0.2	0.5	—	0.8
Sc. faecalis, ATCC 8043	0.5	0.3	—	5.8
Sc. lactis	0.5	0	—	7.5
Sc. liquefaciens, ATCC 4532	0.6	0.8	—	6.8
Sc. thermophilus	0	3.7	0.27	4.3
Sc. salivaricus, ATCC 9758	1.1	1.0	0.03	6.8
Pc. lindneri	1.0	4.7	0.03	4.8

Values are expressed ml of 0.1 N NaOH in ten ml medium after 44 hours incubation.

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[2] K. Yamanaka, This Bulletin, **24**, 305 (1960).

collected and used. Some strains were provided by Dr. K. Ogata, the Institute of Fermentation, Osaka to whom the author is grateful, and the collection number of this Institute (IFO) are expressed in parensis. *Leuc. dextranicum* ATCC 8086, *Leuc. citrovorum* ATCC 8081, *L. plantarum*, *L. arabinosus* ATCC 8008, *L. xylosus*, *L. cucumeris* (3074), *L. saké* (3497), *L. wortmannii* (3075), *Sc. thermophilus*, and *Pediococcus lindneri*.

Other strains such as *L. lactis*, *L. helveticus* ATCC 335, *L. acidophilus*, *L. bulgaricus*, *L. delbrueckii*, *L. casei*, *L. leichmannii* ATCC 4797 (3073), *Sc. faecalis* ATCC 8043, *Sc. lactis*, *Sc. liquefaciens* ATCC 4532 and *Sc. salivaricus* ATCC 9758 were also used. But these strains could not be shown to ferment pentose, so the production of isomerase was not studied. The methods of experiments, culture of bacteria, preparation of enzyme, enzymatic assay and determinations were carried out in the same way as mentioned previously2).

RESULTS

Acid Production from Pentoses

Growth on pentoses (D-xylose, L- and D-arabinose) and on glucose was determined by titration with a standard alkali solution, and values were expressed as the ml of N/10 NaOH consumed to neutralize 10 ml of medium containing 1 per cent sugar after 40 hours' incubation. The results obtained are listed in Table I. Among thirty-three strains of lactic acid bacteria, nine strains of heterofermenters could grow on both D-xylose and L-arabinose, two strains of heterofermenters (*L. fermentum* and *L. fermenti*) and a homofermenter *L. xylosus* showed growth only on D-xylose, eight strains of homofermenters grew well on L-arabinose. However, poor or no growth and no acid production were obtained with two strains of Leuconostoc (*Leuc. dextranicum* and *Leuc. citrovorum*) and other strains of homofermenters (lactobacilli and streptococci). D-Arabinose was not fermented by all strains even by L-arabinose-fermentable strains. As already mentioned, it was confirmed that D-arabinose has not been isomerized and fermented by lactic acid bacteria.

Specific Activity

Specific activity of pentose isomerase was expressed as μM of ketopentose per mg of protein from aldopentose in 10 minutes' incubation under the system noted above, and the results are shown in Table II.

TABLE II. SPECIFIC ACTIVITIES OF PENTOSE ISOMERASE

	Organism	D-Xylose isomerase	L-Arabinose isomerase
Heterofermenters	*L. fermentum*	4.12	—
	L. fermenti	1.0	—
	L. brevis	1.05	2.40
	L. pentoaceticus	2.45	2.40
	L. gayonii	2.44	1.76
	L. mannitopoeus	2.64	2.83
	L. lycopersici	3.1	1.80
	L. buchneri	4.60	2.83
Homofermenters	*L. xylosus*	5.60	—
	L. plantarum	—	1.86
	L. cucumeris	—	3.17
	L. arabinosus	—	3.18
	L. saké	—	3.62
	L. wortmannii	—	3.38
	Pc. lindneri	—	3.81
	Sc. thermophilus	—	3.77

Effect of Sugar for Carbon Source on Isomerase Production

Growth on other sugars such as fructose, mannose and galactose by lactic acid bacteria was examined. These sugars are then used as a single carbon source. Changes of activity of pentose isomerase are tested on the extracts prepared from the cells which are thus obtained. A remarkable difference on the isomerase activity by the two types of lactic acid bacteria (homo- and heterofermenters) employed was demonstrated. The results are listed in Tables III, IV and V. Incubation was proceeded at thirty minutes, and activity was expressed as micromoles of ketopentose formed under such experimental conditions. From L-xylose and D-arabinose, ketose was never obtained in all strains and in any of the conditions tested here.

In regard of the homofermenters which were able to ferment L-arabinose (*L. plantarum*, *L. cucumeris*, *L. saké*, *L. wortmannii* and *Sc. thermophilus*), activity of L-arabinose isomerase was

TABLE III. EFFECT OF CARBON SOURCE ON PENTOSE ISOMERASE PRODUCTION

		Protein in cell-free extracts	Ketopentose formed (μM) from				Ratio of activity**		
Organism	C-source	(mg)	D-X	L-X	L-A	D-A	XI	AI	XI:AI
L. brevis	glucose	8.0	4.83	—	1.5	0	118	56.3	1: 0.31
	fructose	5.1	2.17	—	0.8	0	53	30	1: 0.27
	mannose*	1.57	1.0	—	1.17	—	24.4	44	1: 1.17
	galactose	1.32	1.05	—	1.29	—	25.6	48	1: 1.23
	xylose	3.14	4.1	0	1.14	0	100	43	1: 0.36
	arabinose	4.4	2.08	0	2.67	0	51	100	1: 1.28
L. pento-aceticus	glucose	1.4	0.68	0	2.3	0	12.1	123	1: 3.4
	fructose	4.6	1.13	0	2.37	0	20.2	127	1: 2.1
	galactose	0.81	0	—	0.44	—	0	23.5	—
	xylose	2.1	5.6	0	1.53	0	100	82	1: 0.27
	arabinose	4.0	0.15	0	1.87	0	3	100	1: 12.5
L. lyco-persici	glucose	9.7	2.26	—	3.05	0	48.5	160	1: 1.35
	fructose	0.44	0.35	—	3.07	—	8	161	1: 8.8
	mannose	1.42	0.78	—	1.87	—	17	99	1: 2.4
	galactose	1.93	0.51	—	0.88	—	11	46	1: 1.72
	xylose	1.4	4.67	0	0.78	0	100	41	1: 0.16
	arabinose	3.7	0.42	0	1.9	0	9	100	1: 4.5
	malt extract	0.13	0.39	—	1.3	—	8	69	1: 3.34
L. mannito-poeus	glucose	2.44	0.53	0	1.8	0	11.1	80.5	1: 3.4
	fructose	2.47	0.45	0	1.31	0	9.5	58.5	1: 2.9
	galactose	8.6	1.28	—	2.37	0	27	105.8	1: 1.9
	xylose	4.65	4.77	0	2.34	0	100	104.5	1: 0.5
	arabinose	3.96	0.8	0	2.24	0	17	100	1: 2.8
	malt extract	3.17	2.1	—	2.8	—	44	125	1: 1.33
L. gayonii	glucose	4.4	1.16	0	2.84	0	31.5	86	1: 2.45
	fructose	4.82	0.76	—	2.07	0	20.9	63	1: 2.6
	galactose	1.3	0.27	0	2.93	0	7.1	89	1: 10.8
	xylose	4.1	3.83	0	2.4	0	100	73	1: 0.63
	arabinose	3.75	0.21	0	3.3	0	5.5	100	1: 15.7
	malt extract	1.8	1.04	—	1.5	—	27.2	45.5	1. 1.45
L. buchneri	glucose	0.28	0.12	—	0.14	—	2.6	—	—
	fructose	2.0	0.7	0	1.54	0	15.1	66	1: 2.27
	galactose	0.63	0.11	—	0.67	—	2.4	28.6	1: 6.1
	xylose	3.7	4.65	0	1.72	0	100	73.5	1: 0.37
	arabinose	3.8	1.1	0	2.34	0	23.7	100	1: 2.12
	malt extract	0.5	0.52	—	2.05	—	—	88	1: 3.95

* 0.1% of glucose was added.

The following abbreviations are used: D-X, D-xylose; L-X, L-xylose; L-A, L-arabinose; D-A, D-arabinose; XI, xylose isomerase; AI, arabinose isomerase.

** Changes of activities of D-xylose- and L-arabinose isomerases were expressed as follow:

Ratio of activity = (micromoles of ketopentose formed by each=extract) / (micromoles of ketopentose formed by extract from pentose grown cells) × 100

TABLE IV. EFFECT OF CARBON SOURCE ON PENTOSE ISOMERASE PRODUCTION

		Protein in cell-free extract		Ketopentose formed (μM) from			Ratio of activity
Organism	C-source	(mg)	D-X	L-X	L-A	D-A	XI
L. fermentum	glucose	0.74	0	—	0	—	0
	fructose*	0.81	0.64	—	0	—	20
	mannose	1.52	3.14	0	0	0	100
	galactose*	0.28	0.06	—	0	—	2
	xylose	1.10	3.14	0	0.1	0	100
	arabinose*	0.55	2.21	—	0.1	0	71
	maltose	5.22	0.25	—	0	—	8
	sucrose	3.75	0.2	—	0	—	6
	gluconate	3.25	0.16	—	0.05	—	5
	malt extract	4.8	3.76	—	0.07	—	120
L. fermenti	glucose	2.15	0.1	0	0	—	2.6
	fructose	0.7	0	0	0	0	0
	mannose	1.47	0.3	0	0	—	7.7
	xylose	1.0	3.9	0	0	—	100
Leuc. mesenteroides	glucose	3.46	0.2	—	0	0	12.5
	fructose	5.0	1.4	—	0.5	0	87.5
	mannose	4.24	1.27	—	0.4	0	79.5
	xylose	3.2	1.6	—	0	0	100
Leuc. dextranicum	glucose	3.15	0.11	—	0	0	
	fructose	0.45	0.02	—	0	0	
Leuc. citrovorum	glucose	4.2	0	0	0	0	
	fructose	6.6	0	0	0.22	0	
	mannose	4.25	0	0	0.49	0	

* 0.1% of glucose was added.

found only on L-arabinose grown cells. Among the homofermenters, D-xylose isomerase by *L. xylosus* and L-arabinose isomerase by *Pc. lindneri* were also found in the cells grown on glucose, fructose, mannose or pentose. Whereas in all cases the heterofermenters showed powerful activities of D-xylose- and L-arabinose isomerases. As *Leuc. citrovorum* and *Leuc. dextranicum* are unable to ferment D-xylose and L-arabinose, the activities of two isomerases can not be found. Similarly, as already mentioned in a previous paper[3], *L. fermentum* and *L. fermenti* can not ferment L-arabinose, and no detectable amount of enzyme for isomerization of L-arabinose is found.

DISCUSSION

The specificity of pentose fermentaion by bacterial cells was first demonstrated by Karström[1]. This author found that in all cases *Leuc. mesenteroides* (*Betacoccus arabinosaceus*) was able to ferment glucose but only L-arabinose grown cells fermented L-arabinose. Karström thus considered the enzymes for fermentation of glucose to be "constitutive" and termed those for L-arabinose as "adaptive". Moreover, in *L. pentoaceticus* he also observed that enzymes for fermenting D-xylose and L-arabinose are constitutive, whereas those for glucose are adaptive. Subsequently, Lampen and Peterjohn[4] reported the fermentative specificity for various

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TABLE V. EFFECT OF CARBON SOURCE ON PENTOSE ISOMERASE PRODUCTION

Organism	C-source	Protein in cell-free extract	Ketose formed (μM) from							Ratio of isomerase activity
		(protein, mg)	D-X	L-X	L-A	D-A	D-R	G	M	
L. plantarum	glucose	2.38	0	—	0	0	0	0	0	
	fructose	1.7	0	—	0	0	0	0	0	
	mannose	2.4	0	—	0	0	0	0	0	
	arabinose	1.06	—	—	2.43	0	—	—	—	
L. cucumeris	glucose	4.15	0	0	0	0	0	0	0	
	fructose	2.25	0	0	0	0	0	0	0	
	mannose	2.25	0	0	0	0	0	0	0	
	arabinose	0.95	—	—	2.40	0	—	—	—	
L. arabinosus	glucose	3.45	0	—	0	0	0	0	0	
	fructose	1.9	0	0	0	—	0	0	0	
	mannose	2.4	0	—	0	—	0	0	0	
	arabinose	1.14	0	0	2.51	0	0	0	0	
L. saké	glucose	1.06	0	0	0	0	0	0	0	
	fructose	0.75	0	0	0	0	0	0	0	
	mannose	0.71	0	0	0	0	0	0	0	
	arabinose	0.91	—	—	1.83	0	—	—	—	
L. wortmannii	glucose	3.22	0	0	0	0	0	0	0	
	fructose	2.6	0	0	0.2	0	0	0	0	
	mannose	2.4	0	0	0	0	0	0	0	
	arabinose	1.10	—	—	2.4	0	—	—	—	
Sc. thermophilus	glucose	4.85	0	—	0.2	0	—	0	0	
	fructose	2.22	0	—	0	0	0	0	0	
	mannose	2.71	0	—	0	—	0	0	0	
	arabinose	2.65	0	—	2.47	0	0	0	0	
	malt extract	4.55	0	—	2.17	—	—	—	—	
Pc. lindneri	glucose	2.1	0.1	—	3.6	0	0	0	0	166
	fructose	0.94	0	—	1.62	—	0	0	0	75
	mannose	1.95	0.1	—	2.13	0	0	—	0	98
	galactose	2.0	0	—	1.63	—	0	0	0	75
	arabinose	2.0	0	0	2.17	0	0	0	0	100
	malt extract	0.92	0	—	1.5	—	—	—	—	69
L. xylosus	glucose	0.27	3.2	0	0	—	0	0	0	78
	fructose	0.32	3.3	—	0	—	0	—	—	80.5
	galactose*	1.11	0.52	0	0	0	—	—	—	12.7
	xylose	0.62	4.1	0	0	—	—	—	—	100

* 0.1% of glucose was added.
The following abbreviations are used: D-R, D-ribose; G, glucose; M, mannose, and about D-X, L-X, L-A and D-A, see in Table III.

pentoses by *L. pentosus*. These authors found that glucose grown cells are able to ferment glucose but not D-xylose and L-arabinose. Xylose grown cells fermented glucose, D-xylose and D-ribose at the same rate, while the rate of fermentation of L-arabinose was considerable low. Therefore it could be concluded that the enzyme for fermenting D-xylose and L-arabinose are adaptive, while the enzyme for glucose is constitutive. Similar results were also obtained with *E. coli* by Littauer et al[5]. Though D-xylose was fermented by glucose grown cells of *E. coli* after an 80-minute lag-period, it was easily fermented by D-xylose grown cells without a lag-period. Thence it is of much interest to discuss whether the production of enzymes for fermentation of pentose is able to adaptive or constitutive by lactic acid bacteria. This problem, judging from our present knowledge, leads us to the production of enzyme fermenting pentoses, that is to say, it gives an explanation to the question of production of pentose isomerase.

The production of pentose isomerase has hitherto been reported by several workers. D-Arabinose isomerase by *E. coli* which was first demonstrated by Cohen was solely produced from a medium containing D-arabinose[6]. L-Arabinose isomerase was also produced by *L. pentosus*[7,8], *Aerobacter aerogenes*[9] and *E. coli*[5] in which was adapted to L-arabinose. D-Xylose isomerase by *L. pentosus*[10], *Pseudomonas hydrophila*[11,12], *E. coli*[5], *Salmonella typhosa*[13] and *Pasteurella pestis*[14] was only found in D-xylose grown cells. The production of rhamnose isomerase was adaptive to L-rhamnose[15,16], but L-fucose isomerase by *E. coli* was obtained from either D-arabinose or L-fucose grown-cells[17]. Therefore it can be concluded that pentose isomerase is produced adaptively by many bacteria. As an exception, Marshall and Kooi found a new enzyme which catalizes the interconversion of glucose and fructose is produced in the xylose grown cells of *Pseudomonas hydrophila*, but glucose-grown cells did not show such activity[18].

In this case, quite a remarkable difference on the production of pentose isomerase can be newly demonstrated among the genus of the bacteria. In lactic acid bacteria, from the results described in this paper, it can be concluded as follows: L-Arabinose isomerase is constitutive in heterofermenters and in *Pc. lindneri*, and is adaptive in homofermenters. Whereas D-xylose isomerase is constitutive in *L. xylosus*, but isomerase activity is varied upon the kinds of carbon source in heterofermenters. Among the heterofermenters, results on the production of xylose isomerase in *L. fermentum* especially attracted the author's attention. In this bacterium, xylose isomerase was produced from the cells grown on xylose, mannose and even on L-arabinose and fructose. However, glucose-grown cells did not produce this enzyme as just in the same as galactose, sucrose, maltose and gluconate grown cells. Furthermore, the addition of glucose inhibited this production[19]. As glucose is seemed to inhibit the induced formation of pentose isomerase[20], experiments on its formation are now in progress.

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Studies on the Glucanase of *Sclerotinia Libertiana*

Part I. Activity of the Glucanase on Glucans of Yeast and Sclerotia of the Fungus

By Yukio SATOMURA, Masayuki ONO and Juichiro FUKUMOTO

Institute of Polytechnics, Osaka City University

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Preparations of glucan obtained from baker's yeast and sclerotia of *Sclerotinia Libertiana* were found to be completely hydrolysed by enzymes of the *Sclerotinia* fungus. Some differences in the molecular structure of the glucans were found upon examination of the modes of degradation by the successive action of *Rhizopus*- and *Sclerotinia* enzyme preparations of which the former had only a partial hydrolytic effect.

The dissolution of glucan in intact cells of yeast, that could be estimated from the rate of autolysis of the cells, was proved to be insignificant on the action of glucanase alone in the *Sclerotinia* enzyme solution. The combined action of glucanase with lipolytic enzyme in the fungus enzyme solution are shown to promote the solubilization of intact yeasts and sclerotium cells.

INTRODUCTION

The cell wall of various genuses of yeast are known to contain glucan[1]. In regard of baker's yeast glucan is especially known as the primary carbohydrate constituent of the cell wall. Lately, M. Kitahara[2] demonstrated that the main carbohydrate constituent of sclerotia of *Sclerotinia Libertiana* is a glucan mainly containing β-1.3 linkages. Hitherto, a polysaccharide called callose which is widely distributed in various groups of fungi[3], lichens, algae and higher plants has been considered to be a glucan. Enzymes attacking glucan are present in the gut juice of the snail, *Helix pomatia*[4], and in extracts of seaweed[5], barley and certain molds[6]. Eddy and Williamson[4] indicated that the snail enzymes including[7] mannanase, chitinase, lipase etc. are applicable for isolating protoplasts from yeasts. The features of specific enzymes in the enzyme mixture attacking the structural components of cell walls of yeast are yet not clarified.

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Sources of glucanase other than the snail, which can readily dissolve the cell-wall of whole-yeasts still unknown. A *Sclerotinia* fungus extract has been found to possess a high activity of glucanase towards substrate prepared from yeasts or sclerotia of fungus. Some aspects of digestion of glucans in intact cells by the fungal enzymes are described in this report.

EXPERIMENTAL AND RESULTS

Preparation of glucan. From yeast: Pressed baker's yeast was autolysed at 50° for about 3 hrs and after centrifugation, dispersed in 2% sodium hydroxide. The solution was heated in a boiling water bath for about 3 hrs. Subsequently after centrifuging and washing with dilute acetic acid, the residue was digested alternately by Takadiastase and papain. The glucan fraction remained in the insoluble part after washing with hot water. This insoluble part, after hydrolysis with 2 N HCl for 5 hrs, was found to contain 70% glucan as glucose.

From sclerotium: The sclerotium obtained from the bran culture of *Sclerotinia Libertiana*, after drying in air and crushing with a grinder, was washed with 50% alcohol and the supernatant was discarded. The residue was further washed with a large amount of water and the supernatant was again discarded. This water-soluble portion had previously been found to contain a slime consisting of a polysacchride and a peptide. The polysaccharide yielded glucose, arabinose and galacturonic acid on hydrolysis, as shown by paperchromatography[8]. From the insoluble material, glucan was extracted with 5% sodium hydroxide and precipitated as the Cu-salt by the addition of cupric sulphate to the extract. Then, after washing the Cu-salt of crude glucan with 2N sodium hydroxide, purified glucan was obtained by removing the copper with 2N hydrochloric acid and washing repeatedly with hot water. The reducing sugar content of the preparation after hydrolysis was found to be 95%.

Sclerotinia enzyme solutions. S_{Aq}; The S_{Aq} enzyme solution was extracted from the dry powder of a bran culture of *Sclerotinia* with ten volumes of water. S_G: From the S_{Aq} solution, the glucanase fraction was salted out with 0.4 saturation solution of ammonium sulphate, and after dialysis against water for 2 days, it was dissolved into one-fifth of the volume of the original extract. S_{Am}: The extract with ten volumes of 1/20 N ammonium hydroxide was dialysed against 5% carbowax (4000) for 1 day and made up to the initial volume with water. S_{Am}-0.4: This S_{Am} solution was salted out by 0.4 saturation of ammonium sulphate and after dialysis, was dissolved in water to one-fifth of the volume of the initial extract.

Rhizopus enzyme solution. Rhiz: The bran culture of *Rhizopus chinensis* was extracted with three volumes of water. The fraction precipitated by a 0.65 saturation solution of ammonium sulphate was passed through an ion exchange resin and dissolved in water to one-tenth of the volume of the initial extract.

Measurement of glucanase. Two ml of the glucan suspension containing 10 mg of glucose after hydrolysis, 2 ml of phosphate buffer solution (pH 5.2) and 1 ml of enzyme solution were mixed and incubated at 40°C.

Glucanase activity. In this manner, it was found that the S_{Aq} can hydrolyse yeast- and sclerotium glucan almost completely to glucose within 2 hours. Also, it was found that almost all of the total activity of the S_{Aq} solution could be regained in the S_G solution. No difference was observed between the glucanase activity of S_{Aq} and S_{Am}.

Lipolytic and other enzymic activity. Although the aqueous enzyme extract had no lipolytic activity, the extract with ammonium hydroxide possessed substantial activity, and this activity was measured on olive oil, Tween 20 and lecithin. However protease activity was very low.

Mode of Degradation of Glucan by Enzyme Preparations of *Rhizopus* and *Sclerotinia*.

The hydrolysis curves of yeast- and sclerotium glucan by *Rhisopus* and *Sclerotinia* enzyme are shown in Fig. 1. With *Rhizopus* enzyme, only a partial hydrolysis of glucans occurred, not exceeding 20% in yeast glucan and 40% in sclerotium glucan even after 24 hours. In this process, sclerotium glucan became almost completely soluble, while yeast glucan remained approximately 70% insoluble. After the digestion of glucans by *Rhizopus* enzyme, the soluble and insoluble fractions were separated by centrifugation, and then successively submitted to the action of *Sclerotinia* enzyme. The hydrolysis of each fraction began immediately and about 99% of each was hydrolysed. Patterns of spots of saccharides on paperchromatograms during degradation of glucans are shown in Fig. 1. Glucose and three other unknown sugar spots were found in both glucan during hydrolysis with *Rhizopus enzyme*, but these unknown spots gradually disappeared during incubation with the *Sclerotinia* enzyme.

Effects of *Sclerotinia* Enzyme Solution on Autolysis of Yeast.

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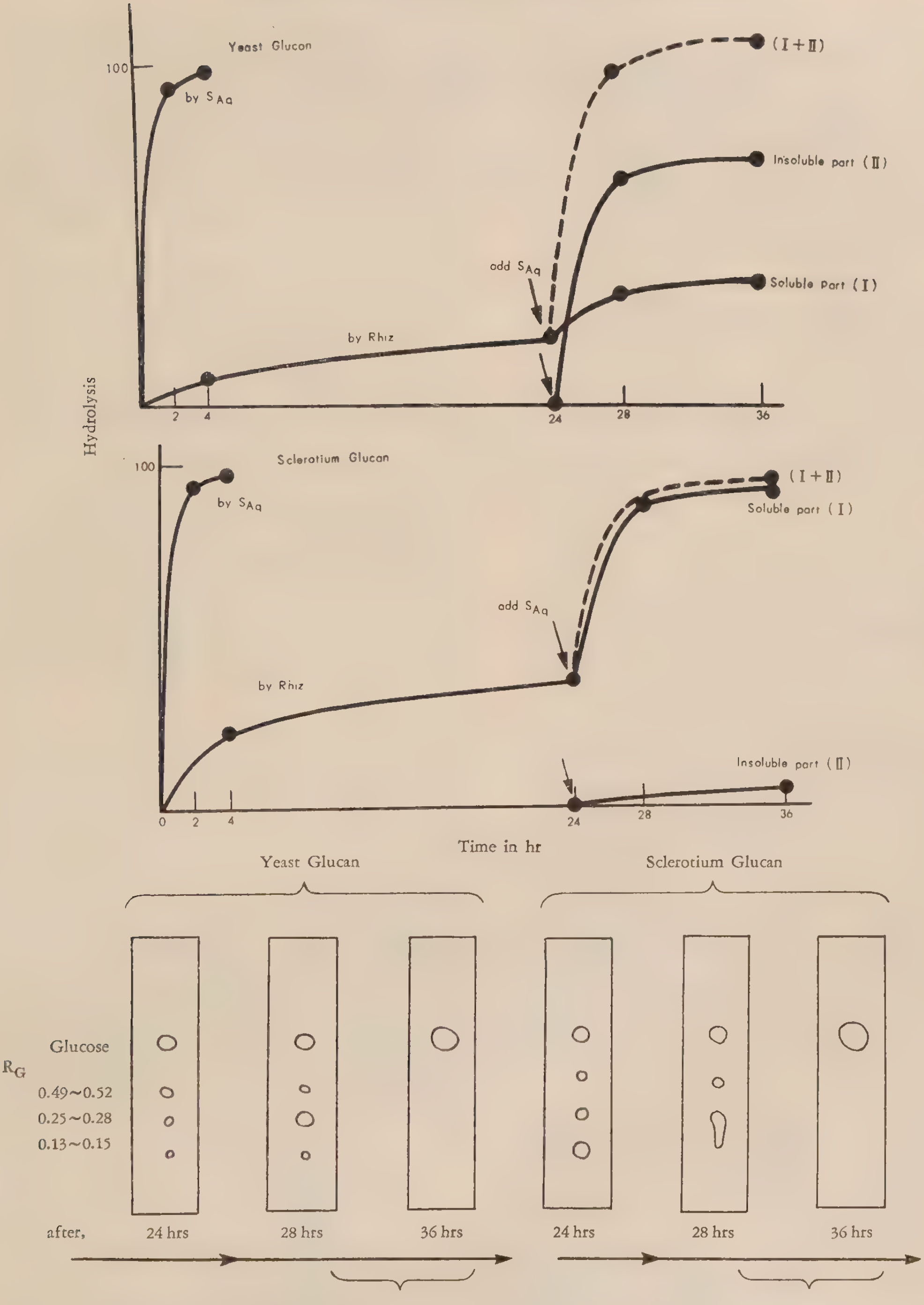

FIG. 1. Degradation of Yeast- and Sclerotium Glucan by *Rhizopus*- and *Sclerotinia* Enzyme Preparations, and Paperchromatograms of the Degradation Products. (Solvent system; *n*BuOH, 4- AcOH, 1- H_2O, 1)

As seen in Table I, the autolysis of baker's yeast was slightly promoted by the *Sclerotinia* enzyme (S_{Aq}). However this effect was neither proportional to the amount of S_{Aq} added, nor was it sensitive to heat treatment. Therefore, this promoting effect had no relation with any enzymatic action of S_{Aq}. Treatment of cells with papain at an appropriate concentration for a comparatively long time, had a considerable promoting effect on the auto-digestion of yeast. Also, alternate successive treatment with papain and S_{Aq} was found to cause dissolution of the cells from 75 to 83%.

Effect of Chemical Pretreatement of Yeast on Digestion of Cells.

As presented in Table II, a mild pretreatment of cells with dilute sodium hydroxide caused much increase in the digestion of yeast by the *Sclerotinia* enzyme solution (S_G). This result may be explained by the assumption that glucan is somehow released from the complex structure of the cell-wall by such treatment and becomes accessible to the action of glucanase. Pretreatment with other reagents such as ethylenediaminetetra-acetate (EDTA), desoxycholate and acetic acid had no effects on the digestibility of cell-wall glucan with the fungus enzyme solution.

Combined Action of Lipolytic Enzyme and Glucanase on Cells of Yeasts and Sclerotium.

The above results suggest that the lipolytic enzyme certainly plays a role in the enzyme of the complex structure of cell wall. A *Sclerotinia* enzyme solution (S_{Am}) containing lipases, phospholipase and glucanase was

TABLE I. EFFECTS OF *Sclerotinia* ENZYME SOLUTION ON AUTOLYSIS OF PRESSED BAKER'S YEAST

8 ml of yeast suspension containing 95.7 mg of dry matter are incubated at 37°C for about 24hrs.

		pH 6.0～5.0	Cells Remained (in mg of dry wt.)	Rate of Autolysis (%)
Control			57.6	40
	Enzyme soln. added			
Cell suspension	$+S_{Aq}$ 1 ml		43.8	54
〃	+ 〃 3 ml		50.1	48
〃	+ 〃 (heated) 1 ml		43.0	55
〃	+Papain 1 ml (2 mg)		32.8	66
Cell suspension	$+S_{Aq}$ 1 ml $\xrightarrow[\text{2 hrs}]{}$ +Papain 1 ml $\xrightarrow[\text{18 hrs}]{}$		23.6	75
〃	+ 〃 (heated) $\xrightarrow[〃]{}$ 〃 $\xrightarrow[〃]{}$		27.8	71
〃	+Papain 1 ml $\xrightarrow[\text{18 hrs}]{}$ S_{Aq} 1 ml $\xrightarrow[\text{2 hrs}]{}$		16.2	83
〃	+ 〃 $\xrightarrow[〃]{}$ 〃 (heated) $\xrightarrow[〃]{}$		31.1	68

TABLE II. EFFECT OF CHEMICAL PRETREATMENT OF BAKER'S YEAST ON DIGESTION OF CELLS BY *Sclerotinia* GLUCANASE SOLUTION (S_G)

2 ml of yeast suspension containing 211 mg of dry matter are pretreated as below and after centrifuging incubated with 1 ml of S_G or S_G heated and 5 ml of dist. water at 37°C for 20 hrs. Digestion of cells by glucanase is expressed as follows; Cells remained with (S_G heated$-S_G/S_G$ heated)$\times 100$

Pretreatment			Cells Remained (in mg of dry wt.)		Digestion of Cells by Glucanase
Agents	conc.	time	with S_G heated	with S_G	(%)
EDTA	M/200	15 min	152.0	152.0	0
Desoxycholate	M/1000	〃	148.0	148.5	0
CH_3COOH	1.5 N	〃	116.5	114.5	0
NaOH	0.15 N	〃	145.0	110.0	24
	after treatment, without centrifuging adjusted pH at 6.0 by HCl				
NaOH	0.1 N	2 hrs	150.0	104.5	30
〃	0.15 N	〃	138.0	66.0	52
〃	0.25 N	4 hrs	82.0	44.0	46

TABLE III. PROMOTION OF SOLUBILIZATION OF YEASTS- AND SCLEROTIUM CELLS BY *Sclerotinia* ENZYME SOLUTION (S_{Am}) CONTAINING LIPOLYTIC ENZYME ACTIVITY

2 ml of yeast suspension are mixed with 2 ml of enzyme solution and 6 ml of dist. water, and after adjusting pH to 6.0~6.4 the mixture is incubated at 37°C for 20 hrs. Promotion of lysis is calculated as follows; Cells remained with $(S_{Am}\text{ heated} - S_{Am}/S_{Am}\text{ heated}) \times 100$

Organisms	Enzyme Solu. added	Promotion of Lysis (%)
Baker's yeast	S_{Am}	17
〃	$S_{Am-0.4}$	30
Saccharomyces cerevisieae	$S_{Am-0.4}$	18
〃 *ellipsoideus*	〃	35
Schizosaccharomyces pombe	〃	24
Candida albicans	〃	38
(Cells were harvested after 3 or 4 days of shaking culture at 25 in dilute wort containg 0.2% pepton)		
Sclerotium of *Sclerotinia* Fungus	$S_{Am-0.4}$	39
	S_G	13

(Sclerotium used was dried in air and crushed with a grinder.)

therefore used to examine the effect on solubilization of intact cells of several varieties of yeast and sclerotium. The promoting effect of the enzyme solution on lysis of the cells was prominently marked as shown in Table III, especially on the cells of *Candida albicans*, *Saccharomyces ellipsoideus* and sclerotia of *Sclerotinia* fungus.

DISCUSSION

The progress of a partial hydrolysis of glucans by *Rhizopus* enzyme may offer important suggestions serving to study on the difference in the branching structure between yeast- and sclerotium glucan. The molecular structure of yeast glucan cannot be regarded as homogeneous. The ends of the molecule are distinguished from the main part which is known to have a brancing structure with 1.2 linkages.[9] At present, M. Kitahara[10] considers that in sclerotium glucan the branching is present at the 1.6 position. The *Sclerotinia* enzyme solution contains the enzymes capable of splitting possibly β-1.2, and -1.6 linkages in yeast- and sclerotium glucan. The *Rhizopus* enzyme probably mainly splits 1.6 linkages.

The complete digestion of cell-wall glucan in intact yeasts and fungal cells could not be attained by glucanase alone, because glucan is considered to be protected from the action of glucanase by combination with protein or lipid.[11] Proteins in the cell walls of micro-organisms are generally resistant to proteases, and lipids are present in various forms. Consequently a special type of protease and a complex of lipolytic enzymes would be necessary to remove such cementing substances in the laminated cell wall. The effect of papain on the digestibility of glucan in intact cells of yeast could only be recognized after prolonged application of the enzyme. However the effect of lipolytic enzymes of the *Sclerotinia* enzyme solution seemed to increase the digestibility of cell-wall glucan immediately, thus releasing the complex structures of the cell wall. The integrity of the cell-wall structure varied to a certain extent with the growth condition of organisms. Hence, complete digestion of cells by the enzyme solution would need further investigation on growth conditions of substrate organisms. Studies on the influence of the enzyme solution on the fermentation of yeast are now in progress.

The authors wish to express their sincere gratitude to Prof. I. Yamasaki for his criticism. They are also indebted to Mr. K. Sakage for his assistance in part of this work.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 322~323, 1960]

Interconversion between the Azoxyglycosides by Cycad Emulsin*

Studies on Some New Azoxyglycosides of *Cycas revoluta* Thunb. Part III

Sir:

Previously, we reported the isolation of cycasin[1] and two β-glucosyl-cycasin, neocycasin A[2] and B[3], which were proved to be β-laminaribiosyl- and β-gentiobiosyl-oxyazoxymethane, respectively. We adverted also to the existence of some other azoxyglycosides in the seed of Japanese cycad.

Here, the evidences are presented that these glycosides are interconvertible by catalysis of cycad emulsin, and further a new higher oligoglycoside, neocycasin C, is also produced. In this case, it is noteworthy that neocycasin A, containing a β-1·3 glucosyl linkage, is predominantly produced from cycasin as substrate, although in the hitherto reported transglucosylation[4,5] a β-1·6 or 1·4 linkage was mainly formed by emulsins obtained from various origins.

The cycad emulsin employed here is an acetone-dried preparation obtained from the cycad seeds by the tannic acid precipitation method[6].

(1) Enzymatic cleavage of neocycasin A or B into cycasin by cycad emulsin was examined as follows. The reaction mixture contained neocycasin A (0.012 M in final concentration), the enzyme (5 mg) and acetate buffer (pH 4.6) in a total volume of 2 ml, and was incubated at 30°C. Neocycasin B was examined in the same way but on an one tenth scale. The intermediary products identified by paper chromatography were cycasin, glucose and laminaribiose or gentiobiose.

(2) The enzymatic transfer reaction was investigated with cycasin as substrate. The reaction mixture, containing 2.5 ml, of cycasin (0.15 M in final concentration), 2 mg of the enzyme and acetate buffer (pH 5.6) was incubated at 30°C under toluene. The samples were withdrawn at intervals, deproteinized and subjected to paper chromatography. As shown in Fig. 1, neocycasin A was the first major transfer product, which was deduced to be synthesized by transfer of β-glucosyl moiety from a donor cycasin to another one acting as acceptor. Laminaribiose, neocycasin B, and later gentiobiose also appeared. Accompanied by further decrease of cycasin or neocycasin A, an appearance of a new azoxyglycoside, neocycasin C, came to be recognized. During the course of this reaction, the accumulation of neocycasin A reached its maximum after nearly sixteen hours.

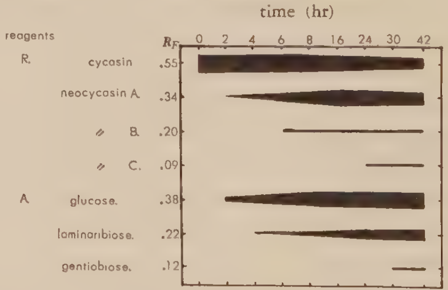

FIG. 1. Paper Chromatogram showing the Fate of Carbohydrates during the Course of Enzymatic Interconversion between the Azoxyglycosides.

Development, n-BuOH-AcOH-H_2O (4:1:1) 2 runs; Reagents, R, resorcin-HCl in aq. EtOH, A, aniline hydrogen phthalate in aq. n-BuOH.

For the conditions of reaction see text.

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* The study was supported in part by the grant to K. Nishida in Aid for Scientific Research from the Ministry of Education.

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2) K. Nishida, A. Kobayashi, T. Nagahama and T. Numata, *ibid.*, **23**, 460 (1959).

3) T. Nagahama, T. Numata and K. Nishida, *ibid.*, **23**, 556 (1959).

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5) H. Suzuki, *Sci. Rep. Tokyo Kyoiku Daigaku*, **B8**, 80 (1957).

6) K. Nishida, A. Kobayashi and T. Nagahama, This Bulletin, **19**, 172 (1955).

(3) Isolation of the reaction products was attempted. A mixture, 10 ml, of the same composition as above was incubated for thirty hours. The deproteinized digest was chromatographed on carbon column (dia. 30×270 mm; carbon 40 g) with water, then aqueous ethanol of stepwise-increasing concentrations successively. The eluates were fractionated into 50 ml portions, of which the carbohydrates were examined by paper chromatography. In the aqueous fractions glucose was found, and in the same way, in the 10% ethanolic ones glucose and gentiobiose, in 15% ones cycasin, laminaribiose, and neocycasin B, in 30% ones neocycasin A, B and C, and in the 40% ones neocycasin C were found. The fractions were separately combined according to their constituents, and were preparatively rechromatographed on paper. The each component separated on the papers was eluted with water, lyophilized, and subjected to the following identification.

The obtained samples and their hydrolysates were all identified by paper chromatography with the respective authentic specimens.

i) Neocycasin A (33.2 mg). After partial acid hydrolysis (0.2 N H_2SO_4, 100°C, 1.5 hrs.), the formation of cycasin, glucose and laminaribiose was proved. Acetylation of the powder gave its acetate in long prisms; m.p. 141.5°~143°C alone or on admixture with authentic heptaacetylneocycasin A.

ii) Laminaribiose (11.3 mg). In the digests with cycad emulsin, glucose was found. Its acetate was obtained as prisms, m.p. 165°~166°C (micro hot plate) which showed no depression on admixture with authentic octaacetyl-β-laminaribiose.

iii) Neocycasin B (3.5 mg). This was cleaved by the partial acid hydrolysis into cycasin, glucose, or gentiobiose.

iv) Gentiobiose (1.9 mg). The degradation products by the complete acid hydrolysis (1 N HCl, 100°C, 1.5 hrs.) was glucose.

v) Neocycasin C (2.4 mg). The chromatographic behavior of this product was the same as that of one of the unidentified azoxyglycosides found in cycad seeds. When it was partially hydrolysed with acid, the spots of neocycasin A, glucose, laminaribiose, cycasin and an unknown sugar were recognized. The last one was considered to be composed of three or more glucose units from its coloration and R_F value. Since any biose or bioside other than the two above mentioned which have β-1·3 linkages was not found, this sugar, too, would have the same configuration. That is, it may be laminari-triose or -tetraose.

Accordingly, the constitution of neocycasin C, though it is not fully established, may be 3-O-β-glucosyl or laminaribiosyl neocycasin A. Further investigations are now in progress.

Tomonori Nagahama
Kotaro Nishida
Tadao Numata

Biochemical Laboratory,
Faculty of Agriculture,
Kagoshima University, Kagoshima

Received December 8, 1959

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Isolation of (+)-Neoiso-isopulegol, a New Stereoisomer of Isopulegol and Piperitenone from a Variety of *Mentha rotundifolia*

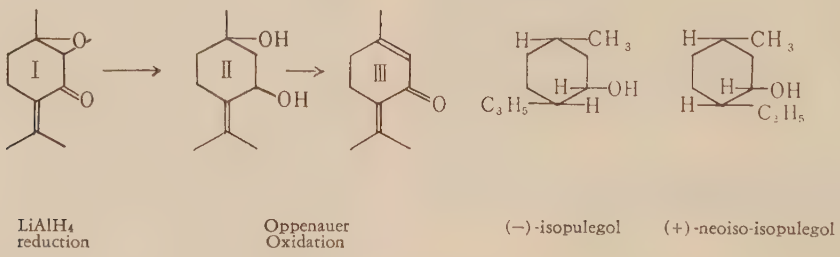
Studies on the Essential Oil of *Mentha rotundifolia*. Part V*

Sir:

In the previous report,[1] one of the authors has established that the *rotundifolia* oils of European origin contain rotundifolone (piperitenone-1,2-epoxide) (I) as the principal component. However, the oil obtained from the French strain** was levo-rotatory and had an absorption at 235 mμ, while the normal oil containing rotundifolone showed highly dextrorotatory value and intense absorption at 260 mμ.

The carbonyl fraction separated from the French strain oil by bisulfite method, had two maxima, 238 and 275 mμ. It yielded at first orange red 2,4-dinitrophenylhydrazone, m.p. 157°C, which gave deep red crystals of m.p. 184°C from a hardly soluble part in methanol. Its analysis coincided well with the value calculated for piperitenone 2,4-dinitrophenylhydrazone; *Anal.* Found for $C_{16}H_{18}O_4N_4$, C, 58.67 H, 5.74, N, 17.02. Piperitenone (III) was prepared by the Oppenauer oxidation of (−)-hydroxypulegol (II) of m.p. 145°C, $[\alpha]_D^{10} -58°$, which was obtained from (I) by lithium aluminum hydride[1], b.p. 105~107°C/10 mm, λ_{max}^{MeOH} 242 mμ (log ε 4.0), and 275 mμ (log ε 3.8). It gave 2,4-dinitrophenylhydrazone of m.p. 184°C and agreed with the value by Naves[2]. It also did not show any depression on admixture with our French strain *rotundifolia* hydrazone, mentioned above.

Besides the isolation of piperitenone, piperitone 2,4-dinitrophenylhydrazone was obtained as orange red needles of m.p. 116°C from the methanol soluble part. The presence of piperitone was also proved by the fact that its semicarbazone, m.p. 218°C was undepressed when mixed with authentic piperitone semicarbazone from a Japanese peppermint oil[3], while piperitenone did not give any crystalline substance by the same treatment[4].



The oil, from which unsaturated ketones were removed by bisulfite method, was fractionated and the fraction, b.p. 68°C/3 mm was treated with excess of 3,5-dinitrobenzoylchloride to give colorless needles of m.p. 91°C in 40% yield. Its analysis coincided with the value for isopulegol 3,5-dinitrobenzoate; *Anal.* Found for

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* This report was presented at the 3rd Meeting of Chem. Perfume, Terpene and Essential Oil at the Univ. of Tokyo on Oct. 20, 1959.; Part IV of this series; S. Shimizu and N Ikeda, This Bulletin, **22**, 201 (1958).

** The seeds of this *Mentha rotundifolia* were imported from Jardin Botanique de Toilouse, France in 1955 and cultivated at Okayama Univ., Japan. This variety was named the French strain in this series, since it showed different aspects as reported previously.

1) S. Shimizu, This Bulletin, **21**, 113 (1957).
2) Y. R. Naves, *Bull. soc. chim.* France, **1951**, 370.
3) S. Shimizu, *Journal of Shinshu Univ.*, No. **4**, 299 (1954).
4) W. Kuhn and H. Schniz, *Helv.* **36**, 167 (1953).

$C_{17}H_{20}O_6N_2$, C, 59.03, H, 5.76. The free alcohol was obtained by digesting benzoate with an alcoholic potash; b.p. 96°C/15 mm, n_D^{10} 1.4775, $[\alpha]_D^{10}+4.04$. Its infrared spectrum showed the characteristic absorption bands for isopropenyl-group, 1640 and 895 cm^{-1}.

Theoretically, isopulegol can exist in four configurations and the three isomers except for *neoiso*-form had been reported by Pickard[5], Read[6], Komatsu[7] and Ueda[8]. However, our substance did not have agreeable constants with anyone of isopulegol isomers, above mentioned. Our isopulegol absorbed 1 mol of hydrogen over Pto_2 in glacial acetic acid to yield viscous oil of menthol-like ordor; 100～105°C/20 mm, n_D^{10} 1.4660, $[\alpha]_D^{10}+0.10°$. Its infrared spectrum was completely identical with that of (+)-neoiso-menthol reported by one of the authors[9] and Naves[10]. Our hydrogenated alcohol also gave 3,5-dinitrobenzoate, m.p. 101°C, which was not depressed by mixture with (+)-neoiso-menthyl-3,5-dinitrobenoate, m.p. 99.5～100.5°C.[9] Since the hydrogenation of isopropenyl group does not concern with asymmetric carbon, the original new alcohol in the *rotundifolia* oil from the French strain can be assigned to be (+)-neoiso-isopulegol. It is well known that natural isopulegol reported by Naves[11] and Penfold[12] and synthetic isopulegols[5,6,13] obtained by cyclization of citronellal in acid media have isopropenyl group at C_4 in *trans* position to C_1 methyl and is related to (−)-menthol. Naves[11] also pointed out that isopulegol occurs only in those oils of which citronellal is also a constituent. On the other hand, isopropenyl group of *rotundifolia* neoiso-isopulegol being *cis* at C_4 and the presence of citronellal was not detected. This fact should not be neglected for investigation of cyclic terpene biosynthesis from acyclic compounds. Furthermore, the French strain *rotundifolia* contains piperitenone but not rotundifolone (piperitenone oxide), which is usually found in many strains of *Mentha rotundifolia.* So it seems to us that the French strain may lack the enzyme catalizing piperitenone to its oxide, which has some connection with neoiso-isopulegol formation. These specific aspects of the French strain are also noteworthy to interpret the essential oil formation in the various *Mentha* plants from a view of biochemical genetics.

Sumio SHIMIZU
Nagamori IKEDA***
Hiroo UEDA****

Laboratory of Agricultural Chemistry,
Shinshu University, Ina, Japan.

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[8] H. Ueda and S. Shimizu, This Bulletin (in preess).

[9] H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

[10] Y. R. Naves and J. Lecomte, *Bull. soc. chim.*, **1955**, 792.

[11] Y. R. Naves, *Parfumes France*, **6**, 191 (1928); **9**, 69 (1931).

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*** Laboratory of Plant Breeding, Okayama Univ., Okayama.

**** Research Institute, Nagaoka and Co., Kobe.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 3, p. 326~327, 1960]

Conversion of D-Glucose to D-Fructose by a Strain of Soil Bacteria

Sir:

Marshall and Kool[1] have described an enzyme in *Pseudomonas hydrophila*, which catalyses the conversion of D-glucose to D-fructose. The authors now wish to report the finding of a bacterial strain which also has glucose isomerising activity. This strain was isolated from soil, and its microbial characters and ability to isomerise D-glucose were investigated.

The characters of the strain were as follows.

Microscopic observation: Rods, 0.5~0.7 by 0.7~1.2 μ, occurring singly. Motile possessing peritrichous flagella. Gram-negative.

Bouillon agar slant: Grayish-white, smooth, glistening, and spreading growth.

Bouillon agr colonies: Circular, smooth, entire, slightly elevated, and grayish-white.

Bouillon broth: Turbid and slight sediment.

Physiological properties:

Temperature: Good growth at 30°~37°C and death at 50°C in 5 min.

Relation to free oxygen: Facultative anaerobe.

Nitrites produced from nitrates.

Catalase: Positive.

Methyl red test negative; Voges Proskauer test Positive.

Citrates utilization in Koser's medium: Positive.

Milk: Coagulation, and partial peptonization.

Gelatin: Slow liquefaction.

Reduction of dyes: Methylene blue and litmus.

Acid and gas from xylose, arabinose, glucose, fructose, galactose, sucrose, maltose, lactose (slow), trehalose, raffinose, mannitol, dulcitol, sorbitol. Acid slightly and no gas from glycerol. Rhamnose, starch, inuline, and inositol not attacked.

According to Bergey's Manual (6th Ed.), it was concluded that this strain related to *Aerobacter cloacae* Bergey et al.

Cells grown in 24 hr. shake culture at 30°C on a synthetic medium[2] with D-xylose as the sole carbon source were harvested with centrifuge. Reaction experiments to D-glucose with washed living cells, lyophilyzed cells and sonic extract were carried out in the presence of arsenate under the similar conditions with the method of Marshall and Kool. In all cases, the formation of one ketosugar was found in the reaction systems, which reacted in the cystein-carbazole test[3] giving a maximum absorption spectra at 560 mμ. The R_F value of the sugar was revealed to be identical with D-fructose and no other sugars were found except added glucose in paper chromatography. The ability of the cells to isomerise D-glucose greatly depended on the sorts of carbon sources in the culture medium, similarly as the investigation by Marshall and Kool, and in the cases of D-glucose and D-fructose as the carbon source, isomerization occurred at very low rate or not.

The ketosugar formed in the reaction system was isolated and confirmed as D-fructose by characterization. In one experiment, the reaction mixture was: Glucose 18 g, $Na_2HAsO_4 \cdot 7H_2O$ 936 mg, $MgCl_2$ 203.3 mg, washed living cells 1.5 g (as dry matter), pH 7.8 and final volume 100 ml. The mixture was incubated at 38.5~39°C in a closed flask. After incubation for 21, 45, and 68 hr, contents of D-fructose determined by the cystein-carbazole method were 17.1, 30.0, and 33.4 per cent respectively on the base of added glucose. The mixture was then deproteinized with $HClO_4$ and deionized by ion exchange resins, Amberlite IR-120 and Dowex-1. Resulted solution was concentrated in vaccum and D-fructose was isolated as the insoluble Ca complex. Then crystallization was carried out in usual way from acetic acid solution and yielded 1.3 g sugar. Recrystallized products from aqueous ethanol had the following properties: $[\alpha]_D^{20}=-91.2°$ (lit. $-92.0°$); m.p. 100.5°~103.5°C (lit., 102°~104°C). Further investigations are in progress.

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The authers wish to express their sincere thanks to Dr. T. Yabuta, Dr. K. Sakaguchi, and Prof. Y. Sakurai of the Univ. of Tokyo, for their encouragement and interest, and also to Mr. K. Komagata of the Institute of Applied Microbiology, Univ. of Tokyo and Mr. H. Machida of the Univ. of Tokyo for their helpful advice in this work.

Nobuzo TSUMURA
Tomotaro SATO

Food Research Institute,
Ministry of Agriculture and Forestry, Tokyo

Received May 29, 1960

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Total Synthesis of *dl*-Deguelin

Sir:

The Houben-Hoesch' condensation of dihydro-β-tubanol[1] (I) (0.8 g) with methyl 4,5-dimethoxy-2-cyanomethylphenoxyacetate[2] (II) (1.6 g) gave a pale brown resinous matter (0.6 g), which was able to be separated by fractional recrystallization from aqueous methanol into dihydrodeguelic acid[3] (60 mg) (III), m.p. 147~148°C, and dihydroisodeguelic acid[3] (IV) (250 mg), m.p. 188°C. III (500 mg) was dissolved in a mixture of acetic anhydride (10 ml) and glacial acetic acid (0.5 ml) and refluxed for twelve minutes in the presence of anhydrous sodium acetate (200 mg). On addition of water (20 ml) brown crystals (200 mg) were obtained, recrystallized from chloroform-ethanol (2:3) to give dihydrodehydrodeguelin[4] (V) as faintly yellow crystals, m.p. 262~265°C (decomp.). The mixture of V (500 mg), N-bromosuccinimide (260 mg) and benzoyl peroxide (20 mg) in carbon tetrachloride (500 ml) was refluxed for twenty minutes. In ten minutes the insoluble crystals were gradually dissolved and finally the color of the solution changed suddenly to fluorescent yellowish green. The resulted solution was washed with sodium bicarbonate solution and kept to stand for one hour, bright yellow crystals (300 mg) separated, melted at 216°C (decomp.), insoluble in every organic solvent except chloroform. Its carbonyl absorption band at 1736 cm^{-1} suggests a coumarino-chromon structure such as rotenonone. The filtrate afforded a brown solid (160 mg) on evaporation. To the extract of the solid with hot ethanol (40 ml) 5% ethanolic potassium hydroxide solution (10 ml) was added and allowed to stand over night. The separating crystals were recrystallised from ethanol to give dehydrodeguelin[4] (VI) as pale yellow feather-like crystals (28 mg), m.p. 225~227°C. In dioxane solution VI (2.2 g) was reduced by sodium borohydride (400 mg) to *dl*-deguelol[5] (VII) which was recrystallized from chloroform-hexane and finally aqueous acetone to afford colorless plates (500 mg), m.p. 180~180.5°C, [Found: C, 69.59, H, 6.06. Calcd. for $C_{23}H_{24}O_6$:

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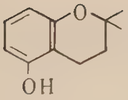
[1] R. Huls, *Bull. Classe Sci. Roy. Belg.*, **39**, 1064 (1953).

[2] A. Robertson, *J. Chem. Soc.*, **1933**, 1163.

[3] S. Takei, S. Miyajima and M. Ono, *Sci. Papers Inst. Phys. Chem. Research*, **12**, 239 (1933).

[4] S. Takei, S. Miyajima and M. Ono, *Ber.*, **66**, 1826 (1933).

[5] According to the private communication, M. Matsui and M. Miyano have performed the partial synthesis of *dl*-deguelin by the oxidation of *dl*-deguelol, prepared from dehydrodeguelin.



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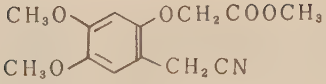

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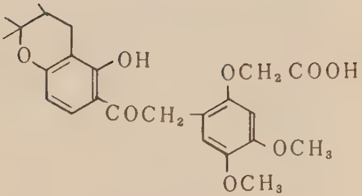

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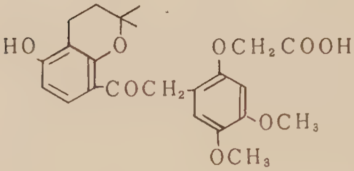

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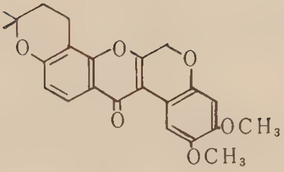

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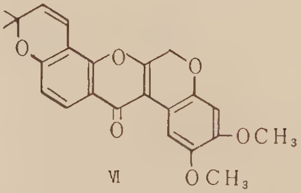

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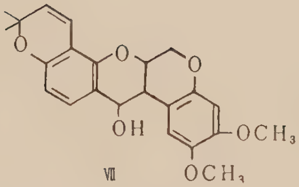
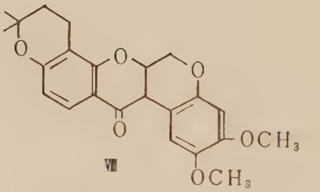


VII



VIII

C, 69.69, H, 6.06%]. The Oppenauer oxidation of VII was carried out in benzene (40 ml) and acetone (30 ml) in the presence of aluminum isopropoxide (3.5 g) under refluxing for ten hours. The oxidation product (200 mg) melted at 167~168°C after recrystallization from ethanol. No depression of mixed melting point with the authentic sample of *dl*-deguelin[4] (VIII) was observed. [Found: C, 70.00, H, 5.70. Calcd. for $C_{23}H_{22}O_6$: C, 70.05, H, 5.58%]. The IR-spectra of the above mentioned synthetic samples, III, IV, V, VI and VIII were identical with the corresponding authentic specimens derived from naturally occurring *l*-deguelin.

We wish to thank Prof. M. Matsui and Dr. M. Miyano for their kind discussions.

Hiroshi Fukami
Junichi Oda
Genzo Sakata
Minoru Nakajima

Department of Agricultural Chemistry,
Kyoto University, Kyoto, Japan

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Studies on the Carotenogenesis of *Rhodotorula* sp. Part I. General Conditions Governing Carotene Synthesis by *Rhodotorula* sp. *Rh*-100 Strain.

(p. *195～198*)

By Masahira NAKAGAWA and Chuji TATSUMI
(*Department of Agricultural Chemisty, Faculty of Agriculture, University of Osaka Prefecture*)

For the purpose of the study of carotenogenesis, a new strain of *Rhodotorula* sp. "*Rh*-100", the major carotenoid of which was β-carotene, was selected. Its power of lipogenesis was strong. The effects of carbon and nitrogen sources on the carotenoid production were investigated and it was found that glucose was the best carbon source but glycerine was not so good and asparagine was the best nitrogen source. The C:N ratio of the medium was an important factor in carotenogenesis and the optimum ratio in *Rh*-100 was 40:1 to 50:1. It was proved that in the glucose medium the carotenoid production of *Rh*-100 increased in proportion to growth, but in the glycerine medium did not so. The addition of glucose to the glycerine medium at the beginning of incubation stimulated the carotenoid production. Light was not essential for carotenogenesis of *Rh*-100.

Studies on the Carotenogenesis of *Rhodotorula* sp. Part II. The Effects of Inhibitors on Carotene Synthesis by *Rhodotorula* sp. *Rh*-100 Strain. (1)

(p. *199～202*)

By Masahira NAKAGAWA and Chuji TATSUMI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Osaka Prefecture*)

The effects of diphenylamine (DPA) on growth and carotenogenesis in *Rh*-100 were investigated and it was found that DPA was inhibitory to both these cases. The idhibition of DPA to growth of *Rh*-100 was almost excluded by the addition of ferric chloride to either glucose or glycerine medium. Then the inhibition of DPA to the carotenoid production of *Rh*-100 could not be excluded by the addition of ferric chloride to the glucose medium, but it could be almost excluded to the glycerine medium. By the addition of proper amounts of phenol, resorcinol and kojic acid to the medium the synthesis of carotenoid in *Rh*-100 was considerably enhanced. It might be that phenolic OH of kojic acid did its work in the process of carotenogenesis.

Polarographic Studies on Storage of Meats. Part V. Relation between Protein Waves of Beef and Changes of its Components. (2)

(p. *202～208*)

By Tetsujiro OBARA and Yasokichi OGASAWARA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been shown in a previous report that the polarographic wave of the protein obtained from the beef varies in its shape and height with the increase in the concentration of some amino acids added. But it is not obvious whether amino acids which are practically free in the beef, greatly affect the protein wave from the extract of the beef. To obtain the fundamental data on the changes of the protein wave from the beef by γ-ray irradiation, we have examined the relation between protein waves of the stored beef and changes of its free amino acids.

The results of the experiment mentioned above are as follows:

(1) In the case of each extract from round, plate ribs and plate, there is the variation in the kinds and amounts of free amino acids in the beef with the increase in storage time.

(2) Of the amino acids detected by us, leucine, valine, alanine and glycine are much free in the beef, while histidine, glutamic acid and aspartic acid are less free.

(3) The free amino acids in the beef detected by us are less. This result indicates that the protein wave from the extract of the beef, is little affected by the free amino acids.

Polarographic Studies on Storage of Meats. Part VI. Influence of Gamma-Ray Irradiation on Beef in Polyethylene Bag. (1)

(p. *208～214*)

By Tetsujiro OBARA and Yasokichi OGASAWARA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

A necessary γ-ray dosage to sterilization of food is about 200×10^4r, and the irradiated foods are preserved well at least for two or three weeks. But some of them decrease their nutritive value and have undesirable odour during the γ-ray irradiation. Therefore, many problems on this point are remained for the sterilization of food by the γ-ray irradiation.

In order to solve these problems we are doing the fundamental study on the changes of the protein wave from the raw beef by the γ-ray irradiation. We have examined the influence of the γ-ray irradiation on the raw beef, which was vacuum-packed into polyethylene bag. The denaturation of protein contained in the beef was examined by polarographic and other methods.

The results of the experiment mentioned above are as follows:

(1) With increasing dosage of the γ-ray, there is a definite variation in odour, colour, pH value and amounts of moisture and proteins of the irradiated beef.

(2) The paper chromatogram of the free amino acids or organic acids from the beef is greatly affected by the irradiation of 259×10^4r, but those do not indicate a distinct change in the irradiation dosage less than 70.3×10^4r.

(3) Free creatinine and creatine in the beef are converted by the irradiation of 259×10^4r, and the third spot coloured by Jaffé reaction is found. This spot is not produced from creatinine. Probably it seems to be formed from creatine. With increasing dosage of the γ-ray, there is the decrease in the amounts of creatinine and creatine in pure solution.

(4) The protein wave from the beef is little affected by the irradiation less than 259×10^4r.

Studies on the Aerobic Sugar Metabolism of Yeast. Part IV. Optimal Temperature, Nitrogen Source and Glucose Concentration for the Gluconic Acid Production by Yeast. (p. *214~217*)

By Shoichi TAKAO

(*Applied Mycological Laboratory, Faculty of Agriculture, Hokkaido University*)

In the previous paper, it has been reported that several strains of *Candida* sp. isolated from fruit produced much gluconic acid from glucose in the culture solution in addition of $CaCO_3$, and among these strains *Candida* sp. C. 37 possess the highest producing ability of gluconic acid.

This paper reports on the optimal conditions of cultural temperature, nitrogen sources and glucose concentrations regarding to the production of gluconic acid examined.

The optimum temperature was 27~32°C. At 37°C, neither the multiplication of yeast cells nor the acid production was discovered.

In nitrogen sources, $NaNO_3$, NH_4NO_3, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, urea and yeast extract besides peptone used in the previous experiments, were examined. Among these nitrogen sources, ammonium salts gave considerably higher yields than peptone. Especially in the culture solution containing 0.09% NH_4Cl, gluconic acid was produced at the rate of highest of 86.96% based on glucose employed for 2 weeks.

Five concentrations of glucose, 5, 10, 15, 20 and 25% were tested; out of which 10% was proved as optimum concentration.

Studies on the Aerobic Sugar Metabolism of Yeast. Part V. Gluconic Acid Production by Shaking Culture. (I) (p. *218~220*)

By Shoichi TAKAO

(*Applied Mycological Laboratory, Faculty of Agriculture, Hokkaido University*)

Previously, it has been reported that *Candida* sp. C. 37 produced a large amount of gluconic acids; its yield was nearly 90% based on glucose employed under the best conditions of still culture for 14 days.

It has been well known that the production of gluconic acid by microorganisms was caused by the extremely aerobic metabolism in which glucose was directly oxidized to gluconic acid.

Therefore, in this experiment, the selection of strains which produce much gluconic acid in shaking culture was carried out.

As a result, of five strains of *Candida* sp. whose gluconic acid producing abilities were very high in still culture, *Candida* sp. C. 52 was found as the best strain; moreover it was proved that the strain produced more gluconic acid than C. 37 which once was the best one in still culture.

Candida sp. C. 52 produced and accumulated much gluconic acid in the yield of about 76% and over 100% based on glucose employed after 2 and 3 days respectively.

Studies on the Variation of the Free Amino Acid Contents in the Seeds of Pea and Broad Bean during the Course of Ripening. (p. *220~223*)

By Ayako MATSUSHITA

(*Department of Nutrition, Kumamoto Women's University*)

A detailed investigation has now made of the variation of the free amino acid contents in the seeds of pea and broad bean during the course of ripening by the combined use of ion exchange chromatography and paper partition chromatography.

Total free amino acids adsorbed on a column of Amberlite-IR-120 (H) (2×25) were thoroughly eluted with aqueous ammonia (5%), and the combined eluate was taken to dryness in vacuo, after which the residue was dissolved in 10 ml of distilled water.

A volume of 0.001 ml of the solution was chromatographed on a sheet of paper by the two-dimensional ascending technique, using 0.1% aceton solution of ninhydrin as a chromogenic spray reagent.

Each spot of chromatograms of amino acids was cut off, and to this was added 2 ml of 0.1% of aqueous solution of ninhydrin and 1 ml of 10% aqueous solution of pyridin, and the solution was boiled on a water-bath for 25 min.

Each of the colored elutes was subjected to spectrophotometrical determination at 570 mμ (in the case of proline and hydroxyproline at 440 mμ)

The results of experiment were tabulated in Table III.

Studies on Shinshiki Shoyu (Semi-Chemical Soy Sauce). Part II. On the Content of Furfural and Acetaldehyde. (p. *223～228*)

By Takahiro UENO and Akio NOBUHARA

(*Noda Soy Sauce Co. Ltd.*)

Carbonyl compounds, obtained from the acidic (non-neutralized), neutralized and fermented solution of the diluted HCl hydrolysate of defatted soybeans by extracting with ether at room temp., pH 7.0 were separated into fractions by the use of liquid chromatography on alumina as 2,4-dinitro phenyl hydrazones.

Furfural, 5-methyl furfural, hydroxy methyl furfural, and acetaldehyde were identified by paperchromatography, melting point determination, ultra violet and infrared spectra.

Furfural, content at the end, point of hydrolysis was about 600 mg/l and this is approx. 92% of total aldehydes.

After neutralizing at 80°C, the furfural content decreased to about 2 mg/l and acetaldehyde was formed. The content of acetaldehyde was about 23 mg/l and this is approx. 36% of total aldehydes.

The acetaldehydes content of the fermented hydrolysate increased to about 120 mg/l and this is approx. 88% of total aldehydes. Furfural content was similar to neutralized solution.

Studies on Shinshiki Shoyu (Semi-Chemical Soy Sauce). Part III. Isolation and Identification of Volatile Carbonyl Compounds. (p. *229～233*)

By Takahiro UENO and Akio NOBUHARA

(*Noda Soy Sauce Co. Ltd.*)

By the steam distillation of 4 kg of neutralized cakes derived from 20 l of diluted HCl hydrolysate of defatted soy beans, 1.5 l of volatile carbonyl compound fraction were isolated.

Fractionation of 3.6 g of 2,4-dinitro phenyl hydrazones of the carbonyl compounds gave 2.25 g of the chloroform soluble mono carbonyl fraction, 0.05 g of the chloroform insoluble polycarbonyl fraction.

From the polycarbonyl 2,4-dinitrophenyl hydrazone fraction, 50 mg of diacetyl-bis-2,4-dinitrophenyl hydrazone were obtained by direct crystallization from hot nitro benzene solution.

The mono carbonyl-2,4-dinitrophenyl hydrazone fraction was separated into fractions by liquid chromatography on silicic acid and celite mixture, reported by Gordon et al., as the adsorbent.

Isovaleraldehyde, acetaldehyde, phenylacetaldehyde, furfural and 5-methyl furfural were identified.

Studies on the Browning Compounds of Broad Bean. Part I. On the Isolation and Identification of Browning Compounds in Green Pod of Broad Bean. (p. *233～237*)

By Toshizo NAGASAWA

(*Section of Rural Home Economics, National Institute of Agricultural Sciences*)

In order to seek the compounds participating in browning phenomenon of green pod of broad bean, four extract-fractions of the tissue were prepared.

In the one-dimensional paperchromatography (solvent system; *n*-BuOH-AcOH-Water=4:1:1, developing reagent: Folin-Denis' phenol reagent and ammoniacal solution of silver nitrate) of these extracts, five spots were developed by the spraying reagents.

Among them the two spots with R_F-value 0.23 and 0.75 showed prominent color intensity, while the rests were in rather faint ones.

The substance indicating the spot of R_F-value 0.23 was isolated, crystallized, and identified as dopa.

The substance of R_F-value 0.75 was not crystallized, but this was presumed as chlorogenic acid by ultra violet absorption spectrum and other qualitative reactions.

Studies on the Browning Compounds of Broad Bean. Part II. On the Distribution of Browning Compounds in Tissue of Green Seed and the Identification of Tyrosine and Dopa. (p. *237～239*)

By Toshizo NAGASAWA

(*Section of Rural Home Economics, National Institute of Agricultural Sciences*)

The localization of polyphenolic substances participating to the browning phenomenon of green seed of broad bean was observed by histochemical procedure.

Sections of seed tissue were treated in sodium carbonate solution of *p*-diazobenzen-sulfofic acid and ammoniacal solution of silver nitrate.

By the observation of these treated sections under microscope, it was recognized that the polyphenolic substances are localized in seed-coat and not in cotyledon.

Therefore, to seek browning compounds in a green seed, the extract of seed-coat tissue was prepared.

In this extract, the presence of dopa and tyrosine were recognized and the latter was isolate and cristallized.

The Change of Casein in Milk at Different Stages of Coagulation by Rennin. (Studies on Milk Coagulating Enzymes. XVIII) (p. *240～245*)

By Kunio YAMAUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Normal skim milk (SM) or skim milk containing added calcium chloride (SMca; SM 50 ml+2 M $CaCl_2$ 3 ml) were ultracentrifuged (78,000 G, 60 min., at low temperature) at different stages of remain coagulation; (1) without rennin, (2) immediately after addition of rennin at 2°C, (3) after standing overnight in the presence of rennin at 2°C and (4) after coagulation occurred by treatment with rennin at 25°C. Rennin activity, nitrogen (total, non-casein, non-protein nitrogen), total calcium and total phosphorus in the supernatant were analyzed. It was estimated that about one half of added rennin was removed by centrifugation probably as a result of combination with casein particles. The remaining activity in the supernatant was essentially the same for the stages of 2～4.

About 10% of total casein was remained in the supernatant of SM even after primary reaction had been complete. This unprecipitable casein was absent in SMca except for the sample of stage 1, in which about 2% was remained. About 1% of non-protein nitrogen and about 4% of non-casein nitrogen were liberated by rennin action. No significant difference was found between stages 1～4 with regard to calcium/nitrogen and phosphorus/nitrogen ratios of precipitate. It was also confirmed that hardening and shrinkage of curd were non-enzymatic reaction unrelated to continuing action of rennin, by experiment using horse blood serum as inhibitor of rennin.

Researches on Ergot Fungus. Part XXXII. Positions of Penniclavine and Triseclavine in the Previously Known Ergot Alkaloids. (p. *246～249*)

By Saburō YAMATODANI and Matazō ABE

(*The Institute for Fermentation, Osaka*)

It was found that elymoclavine yielded on oxidation with potassium bichromate in dilute sulfuric acid a basic substance, $C_{16}H_{18}O_2N_2$, m.p. 221°C (decomp.), $[\alpha]_D=+157°$ (in ethanol), $[\alpha]_D=+153°$ (in pyridine). This substance was identical with penniclavine not only in crystal form, melting point and specific rotation, but also in color reaction, paperchromatographic behavior and IR.-spectrum. Moreover, it has been found that agroclavine also gave on the same oxidation a basic substance, $C_{16}H_{18}ON_2$, m.p. 232°C (decomp.), $[\alpha]_D=+166°$ (in ethanol), $[\alpha]_D=+174°$ (in pyridine). This substance was identical in all respects with triseclavine.

By these findings, the authors were able to demonstrate the previous presumption that penniclavine and triseclavine might be produced by the fungus through elymoclavine and agroclavine, respectively.

This paper was communicated in *Bull. Agr. Chem. Soc. Japan*, **19**, 94 (1955).

Researches on Ergot Fungus. Part XXXIII. Isolation of Further a New Water-Soluble Ergot Alkaloid, Molliclavine, from the Saprophytic Culture of the Elymus-Type Ergot Fungus. (p. *249～252*)

By Matazō ABE and Saburō YAMATODANI

(*The Institute for Fermentation, Osaka*)

An unknown water-soluble ergot alkaloid, $C_{16}H_{18}O_2N_2$, m.p. 253°C (uncorr. decomp.), $[\alpha]_D=+30°$, $[\alpha]_{5461}=+42°$ (in pyridine), was isolated in very small yield from the saprophytic culture of the ergot fungus parasitic on Elymus mollis Trin. growing in Japan. This unknown substance gave a green color with van Urk's and Keller's reagents, but a deep yellow color with concd. sulfuric acid. The UV.-absorption spectrum of this substance almost agreed with those of agroclavine and elymoclavine.

On catalytic hydrogenation, this substance yielded the dihydro-derivative, which gave colors undistinguishable from those given by agroclavine, elmoclavine or lysergic acid with van Urk's reagent, Keller's reagent and concd. sulfuric acid. This substance was proved to be a new alkaloid, for which the authors have proposed the name molliclavine. It was presumed that molliclavine might be 9-hydroxyelymoclavine, and be produced by the fungus through elymoclavine.

This paper was communicated in *Bull. Agr. Chem. Soc. Japan*, **19**, 161 (1955).

Biochemical Studies of Rice Starch. Part XIII. Starch Degrading Action by the Amylase Fraction in the Seedrice at the Milky Stage. (p. *253～258*)

By Ikuo IGAUE, Susumu MAZIMA and Fumio KURASAWA

(*Laboratory of Biochemistry, Faculty of Agriculture, Niigata University*)

In the amylase fraction prepared from the seedrice at the milky stage by precipitation with lead acetate treatment and fractionation with ammonium sulfate, a starch degrading action was recognized and this chemical property was investigated.

When amylose and amylopectin were attacked by the amylase fraction, the blue or red color of the iodine reaction became colorless and production of reducing sugar reached a final extent of 100 per cent glucose, and enzyme activity against amylopectin was greater than that against amylose.

Optimum action of the enzyme was pH 5.5, 40°C.

It was recognized by paper chromatography that degradation products were glucose, maltose and oligosaccharides at first reaction stage, and only glucose at the latter.

Enzyme activity was stable by treatment at 40°C for 30 minutes between pH 5.5 and pH 7.0 but was inactivated below pH 3.5 by the method of saccharification and iodine color reaction.

Besides, enzyme activity by heat treatment of 65～70°C at pH 6.0 for 10 minutes was inactivated. Furthermore, effect of calcium for heat stability was recognized slightly and it was promoted in 0.025 M calcium concentration and it was little or inhibitory in 0.25 M calcium concentration.

Dialysis against distilled water resulted in reduction in enzyme activity, but dialysis against diluted calcium acetate solution resulted in a two-fold increase in enzyme activity.

Enzyme activity by EDTA treatment dropped about 30 per cent.

Fission products, when dextrin was degraded by the amylase fraction, exhibited mutarotation in negative, namely the amylase fraction might be α-type.

Recovery of starch degrading activity by adsorption treatment with corn starch column was about 40 per cent.

Wijaman reaction of the amylase fraction exhibited positive.

Biochemical Studies of Rice Starch. Part XIV. Maltase Action by the Amylase Fraction in the Seedrice at the Milky Stage. (p. *258～262*)

By Ikuo IGAUE, Susumu HIGASHI and Fumio KURASAWA

(*Laboratory of Biochemistry, Faculty of Agriculture, Niigata University*)

In the amylase fraction prepared from the seedrice at the milky stage, a maltase action, in addition to the starch degrading action, was recognized and these chemical properties and relationships among both enzyme actions were investigated.

When the maltose concentration was lowered, maltose was hydrolyzed completely to glucose by the amylase fraction; while at a higher concentration, a weak transglucosidase action, in addition to the maltase action, was recognized by paper chromatography.

Enzyme activity of both fractions from the "Uruchi" and "Mochi" showed almost the same extent.

Optimum action of the enzyme was at pH 4.4～4.5, 40°C.

By acid and alkaline treatment at 40°C for 30 minutes, enzyme activity was stable between pH 5.0 and pH 7.0 and inactivated below pH 2.0.

Further, enzyme activity by heat treatment at pH 6.0 for 10 minutes, was almost uneffected at temperatures below 55° and inactivated above 70°C.

When the amylase fraction was fractionated by zone electrophoresis with starch block or adsoption treatment with tricalcium phosphate gel, the ratio of maltase activity and starch degrading activity in each fraction was not constant.

Tiselius' electrophoretic analysis of the amylase fraction showed that at least three components were present.

Studies on the Utilization of Hydrol. Part III. The Production of Calcium D-Gluconate by Electrolytic Oxidation of Hydrol. (p. *262～267*)

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Electrolytic oxidation of the hydrol was carried out under the following conditions. Potential difference (direct current): 2～15 volts, current density: 1～5 amp./dm^2, quantity of electricity pased through: 100～125% of the theoretical, electrode: graphite plate. Electrolyte; concentration of hydrol: 21～38%, calcium bromide as a catalyst: 13～20% of hydrol, buffer: calcium carbonate, and reaction temperature: 25～30°C.

When the amount of reducing sugar in the hydrol was assumed as that of glucose in order to calculate the apparent theoretical quantity (I) of electricity required for complete oxidation of hydrol and I was passed through, 88.5∼93.3% of initial reducing sugar was oxidized, ahd finally 89.3∼96.3% of that was oxidized by passing through over 100% of I.

Thus, the yield of calcium D-gluconate based on dry substance of hydrol was found to be 43.5∼77.1%.

Studies on Agricultural Anitibiotic. Folimycin, a New Antifungal Antibiotic produced by *Streptomyces neyagawaensis* nov. sp.

(p. *268∼272*)

By Hiroichi YAMAMOTO and Kôiti NAKAZAWA (*Institute for Fermentation, Osaka*), Satoshi HORII and Akira MITAKE (*Research Laboratories, Takeda Pharmaceutical Industries*)

Streptomyces No. 41895, which was isolated from the soil collected at Takada in Neyagawa City, was found to produce an antifungal substance. The mycelium of St. No. 41895 was hygroscopic and not spiral in form. The characteristics of this strain most resembled those of *St. hygroscopic* group or *St. noboritoensis*, but differed from them in several characteristics. The authers, therefore, assigned St. No. 41895 to a new species, *Streptomyces neyagawaensis*. The antibiotic produced by *St. neyagawaensis* was isolated and named Folimycin. Folimycin occurs as white leaflet crystals and melts at 163∼4°C (decomp.). Its optical rotation is $[\alpha]^{20}_{\mathrm{D}}=-11°$ (c, 1 in ethanol). It does not contain sulfur, halogen. Elementary analysis is: C 61.23, 61.43. H 8.74, 8.92, N 1.40, 1.33. The ultraviolet absorption spectrum: 240 mμ ($E^{1\%}_{1\mathrm{cm}}=480$), 284 mμ ($E^{1\%}_{1\mathrm{cm}}=230$). Folimycin gives positive Fehling, Molisch and conc. H_2SO_4 reactions, but negative ninhydrin, ferric chloride and Sakaguchi reactions. Folimycin is closely related to specifically strong inhibition against phytopathogenic fungi and some yeasts, but no inhibition against bacteria. LD_{50} of Folimycin is 0.36 mg/kg (intraperitoneal infection to mice).

Studies on Alternative Fermentations of Lactic Acid Bacteria: Acetoin Formationun der Aerobic Condition.

(p. *272∼275*)

By Akira ÔBAYASHI, Akira OI, and Kakuo KITAHARA

(*Institute of Applied Microbiology, University of Tokyo*)

Homofermentative lactic acid bacteria, such as *Streptococcus faecalis*, *Sc. liquefaciens*, *Lactobacillus thermophilus*, *L. xylosus*, *L. plantarum* and *L. casei* produced acetoin in the aerated fermentation of glucose. *Streptococci* were found to be most suitable for acetoin formation, for example *Sc. liquefaciens* formed acetoin ($[\alpha]^{20}_{\mathrm{D}}=-67.3°$) in a yield of 39% and lactic acid only 2.6% by weight of consumed sugar. For this type of fermentation we proposed to call acetoin fermentation by lactic acid bacteria.

On the contrary, none of heterofermentative strain employed in this experiment produced notable amount of acetoin.

The intact cells of *Sc. liquefaciens* harvested from shaking culture could metabolize glucose aerobically producing acetoin apploximately according to the following formula:

$$\text{Glucose} + O_2 = \text{Acetoin} + 2\,CO_2 + 2\,H_2O$$

But, on the other hand, the cells which were grown in static culture did not produce acetoin under the same conditions. These results indicate that the ability of acetoin formation from glucose may be induced adaptically under aerobic conditions.

Sc. faecalis also produced acetoin aerobically in a yield of about 30% of consumed arabinose. In static fermentation this strain produced lactic acid in a yield of far more than 60% which is expected from normal pentose fermentation of lactic acid bacteria. A new type of pentose fermentation ought to be proposed.

The Preparation of Soy Bean Hexokinase.

(p. *275∼278*)

By Tatsuro ITOH

(*Department of Agricultural Chemistry, College of Agriculture, Kyoto University*)

Hexokinase activities of soy bean, azuki bean, mung bean and rocambole were determined with glucose as sugar substrate, and soy bean and rocambole (underground stem) proved relatively high activities.

Hexokinase activity of soy bean was increased by germination and its highest activity was found after germination for three days at 26°C. This hexokinase was precipitated with ammonium sulfate at sixty percent saturation. The activity of adenosine triphosphatase, an interfering enzyme for the hexokinase of soy bean was relatively low.

Studies on the Cellulose-decomposing Bacteria in the Rumen. Part IV. About the Bacteriological Nature of the Mesophilic and Aerobic Cellulose-decomposing Bacteria.

(p. *278∼285*)

By Akira AKASHI

(*Department of Zootechnical Science, Faculty of Agriculture, Kyushu University*)

By the methods in the report I∼III, 260 crudely cultured strains of the cellulose-decomposing bacteria were isolated from cows, goats, and sheep, These strains were devided into 9 types by the color produced on the filter paper. These types are as follows.

Type 1. This type is no-color type on the filter-paper. The filter-paper is cut and completely decomposed and this part is dispersed into fibril by shaking.

Type 2. The filter paper is cut and the part under the surface of liquid is dispersed into fibril by shaking and the color of the sediment is cream or orange.

Type 3. The filter paper is cut and the part above the surface of liquid is dispersed into fibril by shaking and the color of the sediment is partically transparent or yellow.

Type 4. The filter paper at the part of the surface of liquid shows slightly yellowish coloration and the color of the sediment is yellow.

Type 5. The filter paper is cut or not cut by shaken and color of the filter paper and or of the sediment is brown or yellowish-brown.

Type 6. The filter paper is cut or not cut by shaken and the color of the filter paper is yellow.

Type 7. The filter paper is cut or not cut by shaken and shows a red coloration.

Type 8. The filter paper is cut or not cut and is dispersed into fibril by shaking and color of the sediment is maroon.

Type 9. The filter paper is cut or not cut by shaken and the color of the sediment is black.

From 260 crudely cultured strains, 35 pure strains were isolated, which were picked up from each type in the rate of 1 or 5 strains, and their characteristics were tested, and they are the genus *Cellvibrico albogilva*, *Cytophaga hutchinsonii*, *Pseudomonas rubeolifacience*, *Cytophaga rubra*, *Cytophaga fulvus*, *Cytophaga krezemieniewskae*, *Cellvibrio vulgaris*, *Sporocytophaga ochracea*, and *Sporocytophaga congregata* var. *maroonicum* n. var.

Studies on the Cellulose-decomposing Bacteria in the Rumen. Part V. About the Distribution in Japan and the Difference in Animals of the Mesophilic and Aerobic Cellulose-decomposing Bacteria. (p. *285～289*)

By Akira AKASHI

(*Department of Zootechnical Science, Faculty of Agriculture, Kyushu University*)

The aerobic and mesophilic cellulose-decomposing bacteria were isolated from cows in every district of Japan. There was no special distribution of these microbes. From every district, the genus *Cytophaga* (in the rate of 40 or 50%), the genus *Cellvibrio* and the genus *Sporocytophaga* were isolated. These microbes were also isolated from the rumen of goats and sheep.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
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Metabolism of Gibberellins by Microbes. Part I.
Isolation and Some Properties of a Gibberellins-Utilizing *Brevibacterium*. (p. *291~295*)

By Shigeo KUBO, Shirō TANAKA and Shukuo KINOSHITA

(*Kyowa Fermentation Industry Co. Ltd., Tokyo Research Laboratory*)

(1) A bacterial strain which utilizes gibberellins as a sole source of carbon was isolated from soil, and was named as *Brevibacterium gibberelium*.

(2) Besides gibberellins, the strain can utilize many sugars, alchols and organic acids, but not starch.

(3) In concentrations from 400 to 1500 γ/ml of gibberellins as a sole souce of carbon, the turbidity of the bacterial culture was paralleled to the concentration of added gibberellins.

(4) The strain degradated gibberellins and decreased the activity of gibberellins for plant elongation.

(5) The enzyme which degradated gibberellins was an intracellular enzyme. Crude enzyme solution obtained from the cells grown on a gibberellins medium did not degradated glucose.

Biochemical Study of Rice Starch. Part IX.
Chemical Properties of Rice Starch prepared from Various Kinds. (2) (p. *295~299*)

By Humio KURASAWA, Ikuo IGAUE and Tosiro HAYAKAWA

(*Department of Agriculture, Niigata University*)

Various kinds of rice of Norin No. 1, Kosiziwase, Aikoku No. 70, Tangin, Rikuto No. 21 were cooked and the qualities of each rice were investigated and then the properties of starches which prepared from various kinds of rice, were investigated.

The cooked rices of kosiziwase, Norin No. 1, Aikoku No. 70 were very sticky. Those of Tangin, Rikuto No. 21 were dry with minium stickiness. The starch from kosizewase, Norin No. 1, Aikoku No. 70 contained 18 ~22 per cent amylose but another starch from Tangin and Rikuto No. 21 contained 24~27 per cent amylose.

Biochemical Study of Rice Starch. Part X.
Chemical Property of Starch of which is not Easy to Precipitate. (p. *299~303*)

By Humio KURASAWA, Ikuo IGAUE and Tosiro HAYAKAWA

(*Department of Agriculture, Niigata University*)

When we prepared starch from white rices (Norin No. 1, Kosiziwase, Aikoku No. 70, Tangin, Rikuto No. 21) with a 0.2 per cent sodium hydroxide solution, we recognized that there are two kinds of starches. One of them is normal rice starch and the other is a starch not as easy to precipitate as normal rice starch. In this report we experimented with the starch which is not easy to precipitate. That is, the starch which is not easy to precipitate was collected by the use of a centrifugal machine and a property of this starch was compared with the normal starch.

The yield of each starch was as follows:

Kind of rice	Normal starch	Starch which is not easy to precipitate
Norin No. 1	76.7%	8.2%
Kosiziwase	75.5	13.0
Aikoku No. 70	70.9	12.8
Tangin	70.0	16.6
Rikuto No. 21	70.5	15.6

The intensity of iodine color reaction of the starch which is not easy to precipitate was lower than that of normal starch.

Amylose content of each starch was as follows:

King of rice	Normal starch	Starch which is not easy to precipitate
Norin No. 1	20.3%	11.3%
Kosiziwase	20.0	13.7
Aikoku No. 70	20.5	12.5
Tangin	24.3	16.3
Rikuto No. 21	26.5	13.7

The intensity of iodine color reaction of amylose in the starch that is not easy to precipitate was greater than the intensity of iodine color reaction of amylose in normal starch.

Potato Glucokinase and Fructokinase.
(p. *303~306*)

By Yoshiyuki INOUYE and Tatsuro ITOH
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University*)

Potato glucokinase was associated with particulate fraction in cell and was sedimented by centrifugation for fifteen minutes at 18,000 ×g, and the fructokinase was presented in soluble fraction. Glucosamine and mannose were phosphorylated by the particulate fraction and were not phosphorylated by the soluble fraction.

These results indicated that the both sugars were phosphorylated by the glucokinase. The glucokinase was partially purified with acetone between thirty five and forty five percent concentration. The phosphorylated products of glucosamine and mannose were studied with this partially purified glucokinase and the results indicated that the products were glucosamine-1-phosphate and probably mannose-1-phosphate.

Soy Bean Hexokinases and their Some Reaction Products. (p. *306～309*)

By Tatsuro ITOH and Yoshiyuki INOUYE
(*Department of Agricultural Chemistry, Faculty of Agricultuye, Kyoto University*)

Soy bean hexokinases phosphorylated glucose, glucosamine, mannose, fructose, galactose and galactosamine. These kinases were separated from each other by fractional pricipitation with ammonium sulfate as glucokinase, fructokinase and a kinase acting on galactose and galactosamine.

When fructose was phosphorylated by the fructokinase, the acid stable phosphorus was formed. Fructose-1-phosphate was not converted to acid stable phosphate by the action of this enzyme preparation. These results indicated that the reaction product of fructose was fructose-6-phosphate.

When glucose, glucosamine and mannose were phosphorylated by the glucokinase, acid stable phosphoruses were formed, and the micromoles of the esterification of these sugars and the formation of acid stalbe phosphoruses were quite agreed during for thirty minutes incubation. From these results, it seemed that the reaction products of these sugars were 6-phosphates. But the enzyme preparations contained weak activity of phosphoglucomutase, so that the products were not detected finally.

Studies on the Carotenogenesis of *Rhodotorula* sp. Part III. The Effects of Inhibitors on Carotene Synthesis by *Rhodotorula* sp. *Rh*-100 Strain. (2) (p. *310～314*)

By Masahira NAKAGAWA and Chuji TATSUMI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Osaka Prefecture*)

α-, β-, γ-, ξ-Carotene and neurosporene had been identified as the constituent carotenoide of *Rh*-100. The effects of diphenylamine (DPA), kojic acid and ferric chloride on the production of these carotenoids were investigated with both glucose and glycerine medium. In the low concentration of DPA, the production of α-carotene was almost inhibited and β-carotene considerably, on the other hand the production of ξ-carotene and neurosporene was enhanced slightly. The production of all these carotenoids in *Rh*-100 increased considerably by the addition of kojic acid to the glycerine medium and flavochrome was produced as the oxidized product of these carotenoids by the addition of ferric chloride to the medium. In the case of the glucose medium, in the presence of ferric chloride, DPA inhibited the production of α- and β-carotene more than DPA alone did, on the other hand in the case of the glycerine medium, in the presence of ferric chloride the inhibitory properties of DPA were excluded and the production of carotenoid increased in the low concentration of DPA.

Studies on the Carotenogenesis of *Rhodotorula* sp. Part IV. The Relation between Carotene Synthesis and Sterol Synthesis by *Rhodotorula* sp. *Rh*-100 Strain. (p. *314～319*)

By Masahira NAKAGAWA and Chuji TATSUMI
(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Osaka Prefecture*)

In the low concentration of glucose, the production of carotenoids and steroids of *Rh*-100 in the case of the addition of valine or valine+asparagine as a nitrogene source to the medium containing quantitatively insufficient glucose for optimal carotene production but sufficient for growth was twice as much as in the case of asparagine alone. These facts suggested that carotenoids and steroids might be probably synthesized from the same fatty acid of C_5. It was recognized that diphenylamine, which inhibited the carotene production, stimulated the sterol production and that kojic acid, which stimulated the carotene production, inhibited the sterol production, this having no connection with growth in *Rh*-100.

Polarographic Studies on Storage of Meats. Part VII. Influence of Gamma-Ray Irradiation of Beef in Polyethylene Bag. (2) (p. *319～323*)

By Tetsujiro OBARA and Yasokichi OGASAWARA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been shown in a previous report that the irradiated beef before 20°C storage indicates a definite variation in its composition with increasing dosage of the γ-ray. But the protein wave from the beef is little affected by irradiation less than 259×10^4r.

In the present report we have examined the influence of the γ-ray irradiation on the raw beef, which was vacuum-packed into polyethylene bag. The denaturation of protein contained in the irradiated beef, which was stored for 57 days at 20°C, was examined by polarographic and other methods.

The results of the experiment mentioned above are as follows:

(1) All the irradiated beefs examined by us are completely spoiled by the 57 days' storage at 20°C. This result indicates that the thin polyethylene film selected by us is not a suitable vacuum-packaging material.

(2) With increasing dosage of the γ-ray, there is a definite variation in the height of the protein wave from the irradiated beef after 57 days' storage at 20°C.

(3) In the case of either protein wave of the water or 7% NaCl extract from the irradiated beef after 57 days' storage as well as before that, the crossing points from the various NH_3- concentrations is approximately constant without influence by the varing γ-ray dosages in the range of our experiment.

(4) This indicates that the quality of the protein from the water or 7% NaCl extract obtained is little affected by the used γ-ray dosages.

Polarographic Studies on Storage of Meats. Part VIII. Influence of Gamma-Ray Irradiation on Shelf Life, Colour and Protein Content of Beef.

(p. *323~328*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculy of Agriculture, Tokyo University of Education*)

In a previous report we have examined the influence of the γ-ray irradiation on the raw beef which was vacuum-packed into polyethylene bag. From this result, it has been shown that the thin polyethylene film selected by us is not a suitable packaging material in the case of the room temperature storage.

For this reason we have examined once more the influence of the γ-ray irradiation by using the raw beef vacuum-packaged into polyethylene-cellophane bag under the same conditions as in a previous experiment.

In the present paper, we have carried out the experiment on the change in the component of the irradiated beef to obtain the much better explanation for the changes of the protein wave from the beef by the γ-ray irradiation.

The results of the experiment mentioned above are as follows:

(1) The irradiated beef can obtain the very desirable result for the storage life at 20°C. This result indicates that the polyethylene-cellophane film selected by us is a suitable vacuum-packaging material.

(2) There is an increase in the irradiation odour of the beef with increasing dosage of the γ-ray. But there is a decrease in the odour with the increase in storage time.

(3) With increasing dosage of the γ-ray, there is a discolouration developed in the beef. Under the condition of the desirable vacuum packaging, the colour of the irradiated beef is stable for the 20°C storage.

(4) When the water extract from the irradiated beef is kept in air, there is a decrease in the variation of the spectral curve for the chromoprotein, with the increasing dosage of the γ-ray.

(5) The protein content of the water extract from the irradiated beef is approximately constant and does not seem to be influenced by the various γ-ray dosages.

In the case of the protein in the 7% NaCl extract from the irradiated beef, there is a decrease in its amounts with the increase in storage time.

Studies on the Organism Producing Isopropanol from Acetone. Part II. Enzymological Aspects on the Oxido-Reduction between Acetone and Isopropanol.

(p. *328~332*)

By Kazuo HOSHINO

(*Kyowa Fermentation Industry, Co., Ltd.*)

In this paper it has been visualized that the intact cells of *Lactobacillus brevis* var. *hofuensis* catalyzes the dehydrogenation of many alcoholic compounds and the reduction of carbonyl substances. As a result of comparative experiments on the substrate specificities of this system, it was found that secondary alcohols and aldehydes respectively and especially dehydrogenating activities for isopropanol was most remarkable. From these findings present author concluded as follows: oxidation and reduction between isopropanol and acetane may be taken place by a participation of new dehydrogenase which differs from yeast and liver alcohol dehydrogenases.

Studies on the Organism Producing Isopropanol from Acetone. Part III. Studies on the Cultural Conditions for the Accumulation of Oxido-Reduction

Enzyme between Acetone and Isopropanol.

(p. *333～336*)

By Kazuo HOSHINO

(*Kyowa Fermentation Industry, Co., Ltd.*)

In this paper the composition of media and the cultural conditions for the formation of the enzyme concerning to oxidation and reduction between isopropanol and acetne in *Lactobacillus brevis* var. *hofuensis* were investigated. As a result, it was demonstrated that the addition of Speakman's inorganic salts and riboflavin to a basal medium (glucose-bouillon medium) resulted in the accumulation of the redox-system. Aerobic cultural condition was also favourable for this purpose. From a medium, however, containing more amounts of iron than Speakman's level, the harvested cells showed weak activity for isopropanol even in above cultural condition.

Studies on the Degradation Mechanisms of Proteins and their Derivatives in the Foods. Part II. On the Heat-labile Sulfur Compounds in Soy-bean Meal Hydrolysates.

(p. *336～339*)

By Yataro OBATA, Yoshinori ISHIKAWA and Junya MIZUTANI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo*)

The heat-labile sulfur compounds which released hydrogen sulfide when it was boiled were absorbed on carbon column from soy-bean meal hydrolysates. It was difficult to elute the sulfur compounds from the carbon column with hydrochloric acid or aqueous phenol solution, however, eluting them with 0.1 N ammonia water was successful. After further purification by cellulose powder column chromatography, phenylalanine and tyrosine were separated, but the separation of the heat-labile sulfur compounds from colored and ninhydrin positive fractions was unsuccessful. Using active alumina column, cystine was removed from the colored and sulfur containing fraction. According to the result of ninhydrin color reaction, the presence of amino group in the sulfur compounds was indstinct. The presence of sulfhydryl or disulfide group in the sulfur compounds could not be confirmed by the spectrophotometric method using *p*-chloromercuribenzoate.

The Effects of Intake Levels of Dietary Protein on the Animals. Part II. Changes in Some Organs, Nitrogen Constituents of Urine and Some Blood Components of Rats fed Low Protein Diet.

(p. *339～344*)

By Hiroshi NISHI, Takashi NATSUKA and Shizuo HAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Utsunomiya*)

In this experiment following results were obtained.

1. When young rats (body weight 40～50 g) were fed for several months on the low protein diet or diet consisted of grains only, rate of their growth was not enough and the body size was less than normal rats, but weights of some organs per 100 g of body weight, number of red blood corpuscles and albumin : globulin ratio remained within the normal ranges.

2. Several urinary nitrogenous constituents of rats fed diets of various protein levels were determined. In general, an increase of dietary protein level was followed by an increase of total urinary nitrogen, and urea, ammonia and creatinine were higher with higher amounts of total nitrogen in the whole range from low to high protein intake level. But only the amount of allantoin arrived slowly to constant value over the certain protein intake level. Therefore it may be possible to make the amount of allantoin an indicator of optimum protein requirement.

Relation between Protein, Calcium and Phosphorus in Abnormal Milk. Part I. On the Constituents of Milk and Blood.

(p. *344～347*)

By Toyoki MIYABE

(*Faculty of Agriculture, Kagawa University*)

(1) Abnormal milk has natural acidity, but indicated alcohol positive character. In comparison with normal milk, this alcohol positive milk contained no abnormal quantity of protein, but indicated a low degree of distribution of casein. A conspicuous fluctuation of Ca/P ratio was also recognized in lactation period.

(2) From concentration of protein in blood, the cow which produced this abnormal milk was considered to be in malnutrition attended by anaemia due to low contents of total solids, haemoglobin and corpuscles in blood.

(3) Component examination of protein by means of electrophoresis of Tiselius apparatus indicated a decrease of albumin and α-globulin but some increase of γ-globulin and fibrinogen, which may mean a slight hepatitis.

(4) Calcium, especially ion-calcium, was increasing in blood serum of this abnormal cow, while casein was decreasing in its abnormal milk. This may be due to disorbered calcium combination in casein at the time of casein synthesis in mammary gland cells.

Relation between Protein, Calcium and Phosphorus in Abnormal Milk. Part II. Protein Distribution and Calcium and Phosphorus Combination with Casein.

(p. *348～352*)

By Toyoki MIYABE

(*Faculty of Agriculture, Kagawa University*)

(1) Alcohol positive milk was presumed to be a kind of physiologically abnormal milk, apart from bovine mastitis milk, due to the following facts: a decrease of casein contents, a slight increase of globulin and, especially, an increase of albumin or protease and peptone.

(2) Positive milk indicated an increase of ion-calcium, combination capacity of calcium and phosphorus with casein rather increase. Especially combination calcium increased and coagulation of casein became conspicuous in the case of high value of combination Ca/P.

(3) Casein distribution of normal milk is usually more than 76%, but, also in positive milk, was found the case of more than 76%. However, the condition of combination of calcium and phosphorus with casein was abnormal.

Studies on the Metabolism of Azuki Seed Protein. Part I. Protease in Azuki Resting Seed.

(p. *352～356*)

By Satoru AKUNE and Shigeaki TAKAGI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The protease (Azuki Protease) in azuki seed (Phaseolus Angularis) was partially purified and some properties of it were investigated.

The enzyme activity was measured by the optical density in 660 mμ using Folin-Ciocalteus phenol reagent. As substrate, 2% haemoglobin and casein both denatured by 6 M urea were used. Optimum pH of this enzyme was about 4,5 (casein as substrate). This enzyme resisted strongly to various protease-inhibitor and activators, and seemed to be non-SH-protease. Optimum temperature of this enzyme was 40°C, but at this temperature the activities reduced to 72% after 30 minutes. Therefore it was confirmed that the heat resistance of this enzyme may be weak. This protease was active to casein twice as much as to haemoglobin and ovalbumin was scarecely decomposed by this enzyme.

Partial purification was carried out using “salting out,” “dyalysis to much water,” “isoelectric precipitation “and” adsorption chromatography by calcium phosphate gel.” As the result, the protease activities became about 25 times of original extract.

Studies on the Metabolism of Azuki Protein. Part II. On the Role of Azuki Protease in Germination.

(p. *357～360*)

By Shigeaki TAKAGI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

In order to elucidate a role played by azuki protease in germination, a part of which was reported previously, the seeds were germinated in the dark and the bright respectively at room temperature.

And the variation of the protease activities in this young plant and the activities toward azuki seed globulin fraction were investigated.

1) By the germination of azuki seed, activity of azuki protease in the dark was twice as large as that in the bright, and in bright germination P_2 component which differed from azuki protease was secondarily produced.

2) Both azuki protease and P_2 component were found scarecely in a germ and a radicle, but in a cotyledon. The same inclination for these activities were exhibited both in the bright and the dark.

3) Azuki protease did not digest the native azuki seed globulin fraction C_3^{ppt}. But the protease appreciably digests the fraction denatured by 6 M urea, although the digestion exhibited a little lower value than that of casein.

From the evidence obtained here, it was suggested that azuki protease was probably distributed in a cotyledon, was the enzyme which digests the seed protein, and increased the activity as germination.

Researches on Ergot Fungus. Part XXXIV. Isolation of Two New Water-Soluble Ergot Alkaloids, Pyroclavine and Costaclavine, from the Saprophytic Culture of the Agropyrum-Type Ergot Fungus.

(p. *360～365*)

By Matazō ABE, Saburō YAMATODANI, Togō YAMANO and Mitsugi KUSUMOTO

(*The Institute for Fermentation, Osaka*)

Three unknown water-soluble ergot alkaloids ,,Z‘‘, ,,U‘‘ and ,,V‘‘ were isolated from the saprophytic culture of the *Agrogyrum*-type ergot fungus parasitic on the *Agropyrum*, *Trisetum*, *Festuca* etc. growing in Japan. ,,V‘‘, $C_{16}H_{18}ON_2$, m.p. 224°C (uncorr. decomp.), $[\alpha]_D^{18}=+109°$, $[\alpha]_{5461}^{18}=+146°$ (in pyridine), was identical with the sample of isosetoclavine. which A. Stoll sent to us, while ,,Z‘‘, $C_{16}H_{20}N_2$, m.p. 204°C (uncorr.), $[\alpha]_D^{20}=-90°$, $[\alpha]_{5461}^{20}=-105°$ (in pyridine) and ,,U‘‘, $C_{16}H_{20}N_2$, m.p. 182°C (uncorr.), $[\alpha]_D^{20}=$

$+44°$, $[\alpha]^{20}_{5461}=+59°$ (in pyridine) were proved to be new alkaloids, for which the authors have proposed the names proclavine and costaclavine, respectively.

Both pyroclavine and costaclavine were seemed to be isomers with festuclavine, and, therefore, considered to be the reduction products of agroclavine. Isosetoclavine („V") was isolated in very small yield together with a large amount of triseclavine, also, from the previous reaction mixture of agroclavine with potassium bichromate in dilute sulfuric acid. It was confirmed that the unpublished alkaloid, isosetoclavine, was an isomer of triseclavine and that, similar to the latter, it was produced through agroclavine by the oxidation action of fungi.

This paper was preliminarily reported in *Bull. Agr. Chem. Soc. Japan*, **20**, 59 (1956).

Researches on Ergot Fungus. Part XXXV. On Reduction of Agroclavine and Elymoclavine with Sodium and *n*-Butanol. (p. *366～371*)

By Saburō YAMATODANI and Matazō ABE

(*The Institute for Fermentation, Osaka*)

The authors have succeeded in obtaining festuclavine, pyroclavine, costaclavine and a basic substance by the reduction of agroclavine with sodium and *n*-butanol. The basic substance, $C_{16}H_{18}N_2$, m.p. 265～7°C (uncorr. decomp.), $[\alpha]^{30}_{D}=+67°$ (in pyridine), $[\alpha]^{30}_{D}=+112°$ (in $CHCl_3$), yielded festuclavine together with costaclavine on reduction under the same conditions. Moreover, the basic substance itself was obtained in excellent yield by treatment of agroclavine with hot sodium butylate. These facts indicated that the substance corresponded to 6, 8-dimethylergolene-(9), for which the authors have adopted the designation lysergine. When elymoclavine was reduced with sodium and *n*-butanol by a similar procedure as in the case of agroclavine, the reaction mixture gave agroclavine, festuclavine, pyroclavine costaclavine and lysergine, as well as α- and β-dihydrolysergols. All these results made clear the stereochemical constitutions of elymoclavine, agroclavine, festuclavine, pyroclavine, costaclavine and lysergine. At the same time, they gave support to the presumption that agroclavine must be produced by ergot fungus through elymoclavine, as well as the authors' view that like festuclavine, both pyroclavine and costaclavine must be produced through agroclavine by the fungus.

This paper was communicated in *Bull. Agr. Chem. Soc. Japan*, 20, 95 (1956).

Germination Promotant for Plants Seed, produced by Microorganisms. Part V. The Germination Promotant for Rice Plant Seeds, produced by *Streptomyces* sp. S-580. (An addendum) (p. *371～374*)

By Yoshihisa KOAZE

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo.* Present address, *Research Laboratory, Meiji Seika Kaisha Ltd.*)

In the previous paper, it has been reported that Factor-D and Factor-E, isolated from the culture filtrate of *Streptomyces* sp. S-580, have promoted synergistically the germination of rice plant seeds, Fujisaka No. 5, in 10^{-4} M ammonium sulfate solution in a petri-dish with filter papers at 18°C. Subsequently the germination promoting effect of a mixture of substances described above in a concentration of 2×10^{-6} g/ml was examined at 11°C, 15°C and 20°C. As the result, only at 15°C, the germination of rice plant seeds, Hōnen-Sōsei or Shinano-Mochi, were promoted about 40% against controls treated without the mixture at a germination-ratio or germination-power. Furthermore, on several L-prolyl diketopiperazines, glycyl-L-proline anhydride, L-leucyl-L-proline anhydride, L-phenylalanyl-L-proline anhydride and L-prolyl-L-valine anhydride (Factor-E), in concentrations of 10^{-4} M and 10^{-5} M with 10^{-5} M. Factor-D dissolving in 10^{-4} M ammonium sulfate solution, the germination promoting effect for rice plant seeds, Fujisaka No. 5, were examined in a petri-dish with quartz-sand at 18°C. As the result, Factor-E, in concentrations of 10^{-4} M and 10^{-5} M, showed the stem- and root-elongation promotion of 10～20% and L-leucyl-L-proline anhydride, in a concentration of 10^{-4} M, also showed the same promotion of about 10% against controls without L-prolyl diketopiperazines, however the other two showed no promoting effect (these values described above were significant through statistical analysis).

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